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The objective of this fellowship is to characterize the roles which C/EBPalpha and beta play in normal mammary gland development and breast cancer. In general, C/EBPα is predominantly expressed at high levels in terminally differentiated, growth arrested cells and the C/EBPβ isoforms, most notably LIP, are expressed at high levels in actively proliferating cells. LIP can act as dominant negative isoform, which when dimerized with other C/EBP family members suppresses transcriptional activity. Consequently, an increase in LIP levels may lead to excessive cellular proliferation. Overexpression of LIP in the mammary gland of transgenic mice results in ductal hyperplasia. Additionally, overexpression of LIP in TM3 mouse mammary cells results in an increase in proliferation via an increase in the number of cells entering S-phase of the cell cycle. Similarly, transplantation of TM3 cells overexpressing LIP into the cleared mammary fat pads of mice also results in very proliferative out-growths. These observations are consistent with the hypothesis that increased LIP expression may inhibit terminal differentiation and provide a selective growth advantage facilitating tumor progression.

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FOREWORD

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Cynthia C. Johnson 8/2/99

PI - Signature Date

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INTRODUCTION

The failure of a cell to maintain balance between proliferation and terminal differentiation often results in tumor formation. The C/EBP family of DNA binding proteins plays a pivotal role in maintaining this balance by regulating the expression of genes involved in terminal differentiation (1, 5, 6). In general, C/EBPα is predominantly expressed at high levels in terminally differentiated, growth arrested cells and the C/EBPβ isoforms, most notably LIP, are expressed at high levels in actively proliferating cells . LIP can act as a dominant negative isoform, which when dimerized with other C/EBP family members suppresses transcriptional activity. Because of an increased DNA affinity of the LIP isoform, this inhibition can occur even at sub-stoichiometric ratios of LIP/LAP (3). Consequently, an increase in LIP levels may inhibit terminal differentiation and lead to excessive cellular proliferation. Consistent with this hypothesis, we have observed elevated LIP levels in several different mouse mammary tumors. These data, as well as the reported observation that C/EBPβ can directly interact with the retinoblastoma protein, has prompted us to investigate the role of C/EBP in mammary gland tumorigenesis (2, 4). We plan to determine whether the overexpression of LIP in both mammary cell lines and in transgenic mice can alter cell growth and facilitate hyperplasia or tumorigenesis. Additionally, we will investigate the mechanisms by which hormonal factors are involved in this overexpression and how elevated levels of LIP may influence the transactivation potential of other C/EBP family members. We hypothesize that overexpression of LIP in mice may block terminal differentiation, and help facilitate uncontrolled proliferation and tumorigenesis. Finally, we will employ a novel combination of gene knockout and mammary gland transplantation technology to study the role of C/EBPα in regulating terminal differentiation.

The following specific tasks were initially proposed for the 36 months of this proposal. Due to the availability of mice, reagents and/or experimental difficulty, a few tasks were completed earlier than expected and some have taken additional time. The reviewer for our 1998 progress report suggested that we submit a revised statement of work (SOW). In compliance with our reviewer's suggestion, the revised SOW is outlined below.

Technical Objective 1: Generation of two complementary models in which we can study the effects of LIP overexpression on mammary gland development and tumorigenesis. Months 1-36. This requires 150 mice.

Task 1: Construction of the WAP-LIP-WAP construct. (Months 1-6).

Task 2: Generation and screening of transgenic mice which overexpress LIP. (Months 6-18).

Task 3: Generation of a stably transfected mouse mammary (TM 3) cell line which overexpresses LIP. (Months 12-24).

Task 4: Transplantation of LIP overexpressing TM 3 cells into the fat pad of BALB/c mice. (Months 24-30).

Task 5: Analysis of LIP overexpression on mammary gland development in both transplanted BALB/c mice and transgenic mice. (Months 12-36).

Technical Objective 2: Determination of the effects of lactogenic hormones on C/EBPβ isoform expression, post-translational processing and functional activity in a mouse mammary epithelial cell line (HC 11). Months 12-36.

Task 6: Generation of a stably transfected mouse mammary cell line (HC 11) with a multimeric

C/EBP promoter-CAT construct and treatment with lactogenic hormones. (Months 12-24).

Task 7: Analysis of C/EBPβ expression and activity in response to hormonal treatment. (Months 24-36).

Task 8: Correlation of LAP/LIP levels with transactivation activity and investigation of post-translational processing. (Months 24-36).

Task 9: Correlation of the effect of ovarian steroids and prolactin on tumor growth in ovariectomized rats with the LAP/LIP ratio. (Months 18-24).

Technical Objective 3: Analysis of the roles of C/EBP proteins on mammary gland development in a C/EBPα knockout mouse. Months 1-12. This requires 48 mice.

Task 10: Removal and transplantation of mammary glands from recipient C/EBPα mice into host 129 mice. (Months 1-6).

Task 11: Analysis of the development of mammary glands from C/EBPα knockout mice. (Months 6-18).

Task 1	Months 1-6	Completed	See progress report 1997
Task 2	Months 6-18	Completed	See progress report 1997
Task 3	Months 12-24	Completed	See progress report 1998 and 1999
Task 4	Months 24-30	Completed	See progress report 1999
Task 5	Months 12-36	Completed	See progress report 1999
Task 6	Months 12-24	Initiated, technical problems	See progress report 1998
Task 7	Months 24-36	Partially completed	See progress report 1999
Task 8	Months 24-36	Initiated	See progress report 1999
Task 9	Months 18-24	Completed	See progress report 1998 and 1999
Task 10	Months 1-6	Completed	See progress report 1997
Task 11	Months 6-18	Completed	See progress report 1997

BODY

Experimental Methods

Protein Extraction and Western Blot Analysis

Tissue and/or cells were disrupted in RIPA buffer (50mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% desoxycholate, 150 mM NaCl, 1 mM EGTA, 0.2% SDS) containing the following kinase, phosphatase and protease inhibitors; 1 mM $NaVO_3$, 1 mM NaF, 1 mM Na_2MoO_4, 10 nM okadaic acid, and 1μg/ml benzamidine, aprotinin, soybean trypsin inhibitor and antipain. Aliquots of these lysates containing 100 μg of protein were electrophoresed on denaturing SDS 12%-polyacrylamide mini-gels, then transferred to PVDF membranes (Millipore, Bedford, MA) overnight at 75 mA. Blots were blocked 90 min in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% Tween-20) containing 3% non-fat dry milk (Carnation, Glendale, CA) then incubated for 90 min in this solution containing antibodies (0.5 ng/ml) (Santa Cruz) prepared against C/EBPβ. Blot washes consisted of TBST (without milk) three times for 5-10 min each, with agitation. Blots were then incubated for 60 min in blocking solution containing 200 ng/ml biotinylated donkey anti-rabbit

immunoglobulin (Amersham, Little Chalfont, England) and washed. Lastly, blots were incubated for 30 min in blocking solution containing 40 ng/ml streptavidin-horseradish peroxidase (Oncogene Science, Uniondale, NY) and washed as before. Enhanced chemiluminesence (Hyperfilm, Amersham) were used for visualization as per the manufacturer's instructions.

DMBA Administration

DMBA (9,10-Dimethyl-1,2-benzanthracene, Sigma) was dissolved in cottonseed oil by heating to 55°- 65°C. Administration of DMBA to mice was conducted in the dark, by oral gavage, at a dose of 1mg/200µl given twice, 1 week apart. The treatment groups consisted of both LIP transgenic and non-transgenic littermates and all mice were pituitary isografted at least 1 week prior to carcinogen treatment.

Transfection and Maintenance of TM3 cells

TM 3 cells were grown and maintained using HEPES buffered D-MEM/F-12 growth media containing: 2% fetal bovine serum, 10 µg/ml insulin, L-glutamine, 5 ng/ml epidermal growth factor (EGF), and 5 µg/ml gentamycin sulfate. At 20 to 40% of confluence, cells were stably transfected with pCIneo-LIP or pCIneo (as control) using Superfect (Qiagen). Stably transfected cells were cloned using cloning cylinders (PGC Scientifics) and maintained with 0.2 mg G418 per ml growth media.

MTS Cell Proliferation Assay

Five LIP clones and five neomycin control clones were plated in quadruplicate into a 96 well tissue culture format at a density of 2 X 10^5 cells per well. The number of viable or proliferative cells were determined for days 1, 3, 5, 7, 9 and 12 of culture using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay from Promega. After application of the MTS reagent, the cells were incubated for 2-3 hrs at 37°C and absorbance at 490nm was measured using a Dynex Technologies Elisa plate reader. Data were plotted as fold change in growth rate.

BrdU Staining for FACS Analysis

Two LIP clones and two neomycin control clones were plated into 100mm tissue culture dishes at a density of 1 X 10^6 cells per plate. At days 3, 7, 10 and 15 of culture, the cells were pulse labeled for 15 min with 1µM BrdU (1 µl of 10mM BrdU per 1 ml of media) (Amersham Life Science), washed with Hanks and removed from the plate using the enzyme Dispase II (Boehringer Mannheim). The pellet was resuspended in 200µl of Hanks and the cells were fixed by the addition of 70% EtOH while vortexing to avoid cell clumping. Cells were stored at 4°C in 70% EtOH until collection of the last time point. Approximately 4 X 10^6 cells were removed from the initial pellet, incubated for 10 min with 3 ml of pepsin (.04% in 0.10N HCl) on a rocker, at RT. After centrifugation (1200 rpm, 5 min), the pepsin supernatant was aspirated, 3ml of 2N HCl were added to a vortexed pellet, and the mixture was incubated for 20 min at 37°C. After incubation, 6 ml of 0.1M sodium borate was added while vortexing, and the cells were pelleted. After aspiration of the supernatant, 6 ml of PBST-B (PBS with 0.5% Tween 20, 0.5% BSA) were added while vortexing, the cells were pelleted, the supernatant aspirated and 1 ml of PBS containing 1 unit of Dnase free Rnase was added and incubated for 30 min at 37°C. The nuclei were again pelleted, the supernatant removed and 20 µl of anti-BrdU FITC and 100µl PBST were added. The nuclei were incubated for 1 hr in the dark at RT and 3 ml of PBST-B were added while vortexing, the nuclei were pelleted, supernatant apirated and propidium iodide (Sigma) was added for a final concentration of 5 or 10 µg/ml in PBST-B. Nuclei were stored at 4°C overnight and examined one day later by FACS analysis.

Northern Blot Analysis

Total RNA was isolated from the inguinal mammary gland of mice using RNAzol B (Tel-Test, Houston, TX) according to the manufacturers instructions. Total RNA (15µg) was resolved on a 1.2% formaldehyde gel before transfer to nylon membrane Hybond-N+ (Amersham). The

blots were hybridized overnight in Express Hyb (Clonetech) at 68°C with 2 X 10^6 cpm/ml of a 1.5 kb, ^{32}P C/EBPβ cDNA probe prepared using the Prime-a-Gene labeling system from Promega.

Results and Discussion

Technical Objective 1: Generation of two complementary models in which we can study the effects of LIP overexpression on mammary gland development and tumorigenesis. Months 1-36.

Task 3: Generation of a stably transfected mouse mammary (TM 3) cell line which overexpresses LIP. (Months 12-24).

A cDNA encoding the LIP isoform of C/EBPβ was cloned into PCIneo from Promega. This vector was chosen because downstream of the CMV enhancer/promoter region is a chimeric intron composed of the 5'-donor site from the first intron of the human beta-globin gene and the branch and 3'-acceptor site from the intron of an immunoglobulin gene heavy chain variable region. It has been demonstrated that constructs which contain an intron flanking the cDNA insert often yield better gene expression. Another advantage of this vector is that it contains the selectable marker gene, neomycin phosphotransferase, under the control of the SV40 enhancer and early promoter region. TM3 cells were stably transfected with either PCIneo-LIP or the vector without insert (PCIneo, as control) and stable cellular clones were generated. The mouse mammary epithelial cell line, TM3, (derived by Dr. D. Medina at Baylor College of Medicine) was determined to be the best line for our transfection studies because they contain relatively low levels of endogenous C/EBPβ as compared to other mouse mammary cells.

Five, stable LIP clones and five vector controls were randomly chosen and tested for proliferative potential using an MTS cell proliferation kit. The various clones displayed different growth rates, but the clones expressing LIP were on average, twice as proliferative as the control cells (Fig.1). To determine whether this increase in cell number or growth was due to an increase in the number of cells entering S phase, cells were pulse labeled with BrdU at 3, 7, 10 and 15 days of culture and analyzed by FACS analysis. The data confirmed our hypothesis and showed that expression of LIP in TM3 cells facilitates entry into S-phase and DNA synthesis. Interestingly, both the LIP expressing cells and the control cells exhibited similar proliferative profiles during exponential growth (day 3) and early confluence (day 7) (Fig. 2B). However, the LIP expressing cells did not remain contact inhibited and by day 10 of culture, at least 10% of the cells had re-entered the cell cycle and were proliferating as compared with the neomycin control cells (Fig. 2B). The 10% increase in proliferation is quite significant and represents half of the proliferation which occurs during exponential growth (20%). This proliferation was also evident by observation of the multiple foci which had formed in the culture (Fig. 2A). Experiments are currently underway to determine if apoptotic pathways have also been altered in these proliferative LIP expressing cells.

Task 4: Transplantation of LIP overexpressing TM 3 cells into the fat pad of BALB/c mice. (Months 24-30).

In order to determine whether the LIP expressingTM3 cells maintained their proliferative growth profile *in vivo*, both the cells which overexpress LIP and the neomycin control cells discussed in Task 3 were transplanted into the right and left inguinal fat pads of virgin BALB/c mice respectively. Approximately 1 x 10^6 cells were injected into the cleared, mammary fat pad and allowed to grow out for 6 weeks. Upon examination of the glands, it was observed that neither the LIP expressing cells nor the neo control cells grew out into normal, ductal outgrowths. Both cell types grew out as undifferentiated (tumor-like) cellular masses (Fig. 3A). This was not unexpected, as these cells have been maintained on plastic for many passages and have lost their ability to undergo normal differentiation. Although both LIP and control cells formed tumors, the LIP outgrowths were approximately 4 times larger (as determined by wet weight) than the neomycin (control) outgrowths (Fig. 3B). Thus, we have provided evidence from both *in vitro* tissue culture and *in vivo* transplantation studies that overexpression of LIP in mammary epithelial

cells results in an increase in the number of cells entering the cell cycle as evidenced by the increased proliferation and increased cellular growth.

Task 5: Analysis of LIP overexpression on mammary gland development in both transplanted BALB/c mice and transgenic mice. (Months 12-36).

The analysis of LIP overexpression in transplanted BALB/c mice has been discussed in Task 4.

The analysis of mammary gland development in LIP transgenic mice has been discussed in our progress reports of 1997 and 1998. The major conclusions from this lengthy descriptive analysis are as follows:

Overexpression of LIP from the Whey Acidic Protein (WAP) promoter results in cellular proliferation which was detected in the mammary gland during lactation and involution. Intraductal papillary hyperplasia was observed throughout lactation (days 3-18) and is best described as intraductal epithelial infoldings, fingerlike projections and branched arcades (Fig. 4). This papillary hyperplasia was also observed in non-transgenic littermates; however, it was more severe in the transgenic mice. Likewise, involuted glands from multiparous transgenic mice contained abnormalities. Three phenotypes were observed: residual non-involuted alveoli, commonly referred to as hyperplastic alveolar nodules (HANs) (Fig. 5B), HANs with a large amount of intralobular stroma or a desmoplastic response (Fig. 5C) and squamous metaplasia accompanied by lymphocytic infiltration (Fig. 5D). Additionally, one involuted gland was characterized as having all three abnormalities listed above as well as regions resembling Ductal Carcinoma In Situ (DCIS) (Fig. 6A) and invasive carcinoma (Fig. 6B).

Task 7: Analysis of C/EBPβ expression and activity in response to hormonal treatment. (Months 24-36).

The regulation of C/EBPβ expression by glucocorticoid hormones was discussed in our progress report of 1998.

Since C/EBPβ expression patterns change with reproductive status in the mouse, we wanted to determine whether steroid hormones such as estrogen and progesterone regulate the C/EBPβ gene. In addition, because the progesterone receptor knockout (PRKO) mouse has a mammary gland phenotype similar to that of the C/EBPβ knockout mouse, we hypothesized that C/EBPβ is regulated by progesterone. However, Northern blot analysis of 14 week virgin mammary glands from either PRKO or wild type mice show that the loss of progesterone signaling through the progesterone receptor has no affect on C/EBPβ RNA levels (Fig. 7 Lanes 1-4). In contrast, replacement of both estrogen and progesterone in both PRKO and wild type mice elevates C/EBPβ RNA levels (Fig. 7 Lanes 5-8). The data demonstrate that transcription of C/EBPβ does not occur via activation of the progesterone receptor. If progesterone is responsible for the increase in transcription then it must occur via a non-progesterone receptor mediated pathway. Two possible mechanisms that can account for the increase in C/EBPβ gene expression are: progesterone antagonism of the glucocorticoid receptor and/or a direct estradiol effect. Additional experiments are needed to clarify the role of estrogen and progesterone in the regulation of C/EBPβ gene expression at both the RNA and protein levels.

Task 8: Correlation of LAP/LIP levels with transactivation activity and investigation of post-translational processing. (Months 24-36).

This task has been inititated and the preliminary data will be included in a future DOD grant submission.

Task 9: Correlation of the effect of ovarian steroids and prolactin on tumor growth in ovariectomized rats with the LAP/LIP ratio. (Months 18-24).

Previous results for this task were discussed in our progress report of 1998.

In addition to those studies, we have initiated an experiment in which both LIP transgenic and non-transgenic littermates were pituitary isografted and treated with the carcinogen, DMBA (9,10-Dimethyl-1,2-benzanthracene) in order to generate mammary gland tumors. Our hypothesis is that if LIP is increasing the proliferative potential of mammary epithelial cells, then the carcinogen induced tumors might arise earlier or grow faster in the LIP transgenics as compared to the non-transgenic littermates. Thus far, three palpable tumors have arisen and they are all from LIP transgenic mice. The first 2 were observed 19.5 weeks after DMBA administration, and the third was observed 23.5 weeks after DMBA administration. It is expected that palpable tumors will be observed about every three weeks and the estimated window for appearance of the tumors is about five months. Both tumor latency and proliferation will be compared between the two groups with the expectation that the tumors arising in the LIP transgenic mice will be more proliferative and perhaps detected earlier.

SUMMARY

Proliferative diseases such as cancer, are often the result of failure to withdraw from the cell cycle at the G_1 checkpoint. Multiple signal transduction pathways, generated by diverse extracellular and intracellular factors, converge at this restriction point and influence cell cycle progression. This advancement beyond late G_1 is believed to be a result of the phosphorylation and consequent inactivation of the retinoblastoma protein (Rb). Recent studies (2) have demonstrated that Rb directly interacts with and activates all of the C/EBPβ isoforms; however, it is not known how this interaction affects Rb activity. This interaction may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland, and supports the hypothesis that the ratio of C/EBPβ isoforms may play a role in the control of cell cycle progression. Support for this hypothesis comes from the observations that the LIP/LAP ratio is regulated during proliferative phases of both liver and mammary gland development (1, 5, 6).

We have proposed that increased LIP expression may inhibit terminal differentiation, thereby facilitating proliferation and perhaps tumor progression. This hypothesis has been supported by our observation that the C/EBPβ protein isoforms, and in particular, the naturally-occurring dominant-negative LIP isoform, have been detected and are highly expressed in aggressive, poorly differentiated infiltrating ductal carcinomas (8). These correlative studies do not directly address the question of whether LIP overexpression, in aggressive tumors, facilitates proliferation and tumor progression or is simply a net result of the tumorigenesis. However, we have been able to answer this question using a combinatorial approach of clinical correlations, *in vitro* tissue culture and *in vivo* transgenic animal studies. Data from the tissue culture experiments has demonstrated that overexpression of LIP increases proliferation by increasing the number of cells which enter S-phase of the cell cycle. Likewise transgenic mice overexpressing LIP in their mammary glands contained hyperplasias and preneoplastic-like tissue. These observations are consistent with the hypothesis that the C/EBPβ-isoforms play a role in regulating terminal differentiation and cell cycle progression.

We are currently extending our tissue culture studies to examine what effects if any, LIP overexpression has on cell cycle proteins (p21, p27, cyclin D1) and how these changes lead to an increased growth rate in mammary cells. Additionally, in transgenic mice as well as C/EBPβ-knockout mice, we plan to investigate the downstream genomic targets of LIP overexpression and how these genes contribute to the altered mammary phenotype observed. These studies will be crucial to understanding the role that the C/EBPβ-isoforms play in breast cancer.

KEY RESEARCH ACCOMPLISHMENTS FOR 1997-1999

- C/EBPβ-LIP is overexpressed in human breast cancer and is associated with indicators of poor prognosis such as loss of estrogen and progesterone receptors, proliferation and loss of differentiation.
- C/EBPα is not necessary for mammary gland development; however, C/EBPβ is required for normal branching and lobuloalveolar development in the mammary gland.
- Transgenic mice overexpressing C/EBPβ-LIP were generated and show ductal hyperplasia and some evidence of preneoplasia and neoplasia.
- A mammary epithelial cell line (TM3) which stably expresses C/EBPβ-LIP was constructed.
  - *In vitro* studies have shown that TM3-LIP expressing cells are more proliferative than the neomycin control cells.
  - *In vivo* transplantation of the TM3-LIP cells into cleared, mammary fat pads results in outgrowths which are more proliferative than the control neomycin outgrowths.
- C/EBPβ gene expression is regulated by lactogenic hormones. The protein isoforms are down-regulated by glucocorticoids and estrogen/progesterone upregulates the C/EBPβ RNA.

REPORTABLE OUTCOMES

Manuscripts

Zahnow, C.A., and Rosen, J.M. Overexpression of C/EBPβ-LIP in the mammary gland of transgenic mice results in altered development and hyperplasia (In preparation).

Zahnow, C.A., and Rosen, J.M. C/EBPβ-LIP alters cell cycle control in TM3 mammary epithelial cells. (In preparation).

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Presentations

Platform presentation at the Department of Defense Breast Cancer Research Program Meeting, Washington, D.C. (1997).

Employment Received

I have accepted a tenure-track position as Instructor in the Oncology Center at Johns Hopkins University.

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FIGURE LEGENDS

Figure 1. TM3 cells expressing LIP have an increased growth rate. Five LIP clones and five neomycin control clones were plated in quadruplicate into a 96 well tissue culture format at a density of 2 X 10^5 cells per well. The number of viable or proliferative cells were determined for days 1, 3, 5, 7, 9 and 12 of culture. After application of the MTS reagent, the absorbance at 490nm was measured and data were plotted as fold change in growth rate.

Figure 2. LIP overexpression in mammary epithelial cells may prevent contact inhibition. Two LIP clones (LIP 1 & 3) and two neomycin control clones (Neo 3 & 10) were plated into 100mm tissue culture dishes at a density of 1 X 10^6 cells per plate. At days 3, 7, 10 and 15 of culture, the cells were pulse labeled for 15 min with 1μM BrdU, harvested and analyzed by FACS analysis for BrdU incorporation. Panel A shows micrographs (4x) of the TM3 monolayers grown on plastic at days 10 and 15 of culture. Note the presence of foci in the LIP clone 1 (upper panels) and the absence of foci in Neo 10 (lower panels).

Figure 3. Mammary gland outgrowths overexpressing LIP have an increased growth rate. The tumor-like appearance and difference in size of the TM3 outgrowths is visible in the mouse of panel A. Note the increased vascularization serving each outgrowth. (B) Bar graph of the wet weight of the LIP outgrowths (n=4) vs. the Neo outgrowths (n=7). The LIP outgrowths are approximately 4 times larger as determmined by wet weight.

Figure 4. The lactating glands of LIP transgenic mice contain papillary hyperplasias. Hematoxylin and eosin stained (5 μm) sections of 10 day lactating mammary glands from two different lines of LIP transgenic mice. Intraductal, papillary, fingerlike projections characterised by a fibrovascular core are represented in panels A and C (see arrows). Panel B depicts a duct with a hyperplastic region containing multi-layered epithelial cells. Panel D is a lower magnification micrograph showing the extent of the papillary hyperplasia along a duct and the abundance of epithelial arcades, branched as well as unbranched.

Figure 5. LIP transgenic mice do not completely involute. Hematoxylin and eosin stained (5 μm) sections of involuted (> 3 months) multiparous, mammary glands. Panel A represents a typical involuted gland from a non-transgenic sibling. Note the sparsity of alveoli and abundance of adipose tissue. A hyperplastic alveolar nodule (HAN) is shown in panel B. Many residual alveoli are present. This is an indication of the lack of regression or an increase in lobulo-alveolar proliferation. Panel C also contains non-involuted alveoli; however, the large amount of intralobular stroma is evidence of a desmoplastic response. Panel D displays a rather advanced squamous metaplasia, in which columnar epithelial cells are changing into squamos epithelial cells. The squamous cells are generating a large amount of keratin (pink concentric material) which has expanded beyond the cellular boundaries and is spilling into the intralobular space. This keratin is probably responsible for the recruitment of the lymphocytes (dark purple cells) which have infiltrated into the lobule.

Figure 6. LIP transgenic mice show evidence of preneoplasias. Hematoxylin and eosin stained (5 μm) sections of involuted (> 3 months) multiparous, mammary glands. A Ductal Carcinoma In Situ-like (DCIS) lesion is shown in panel A. The alveoli or ducts have become expanded by the proliferation of epithelial cells. Note the cribiform pattern produced by the growth of the epithelial arcades. Panel B represents a large desmoplastic response which appears to contain scattered, non-organized epithelial cells which may represent an invasive carcinoma.

Figure 7. C/EBPβ mRNA is not regulated by a mechanism proceeding via the progesterone receptor. Northern blot analysis of 15 μg of total RNA from the mammary glands of 14 week old virgin progesterone knockout (PRKO) mice (lanes 3-4 & 7-8) or 14 week old virgin, wild type, littermates as controls (lanes 1-2 & 5-6). Lanes 1-4 represent untreated virgin mice and lanes 5-8 represent virgin mice treated with estrogen (2 μg) and progesterone (2 mg) for 21 days. Cyclophilin is included as a loading control.

APPENDIX A (Figures)

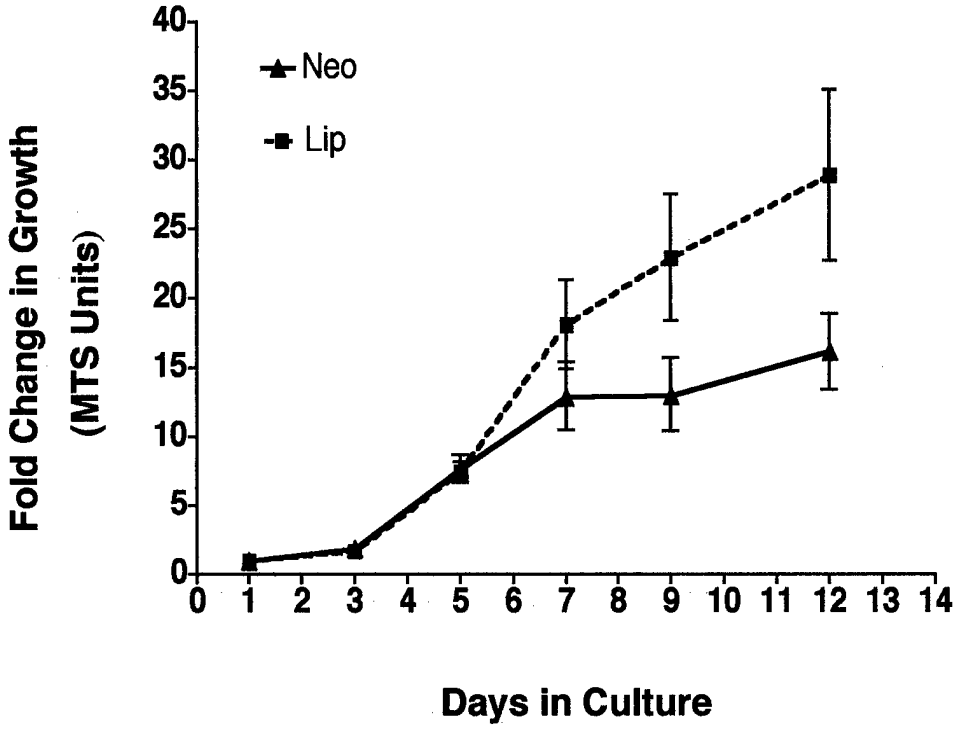
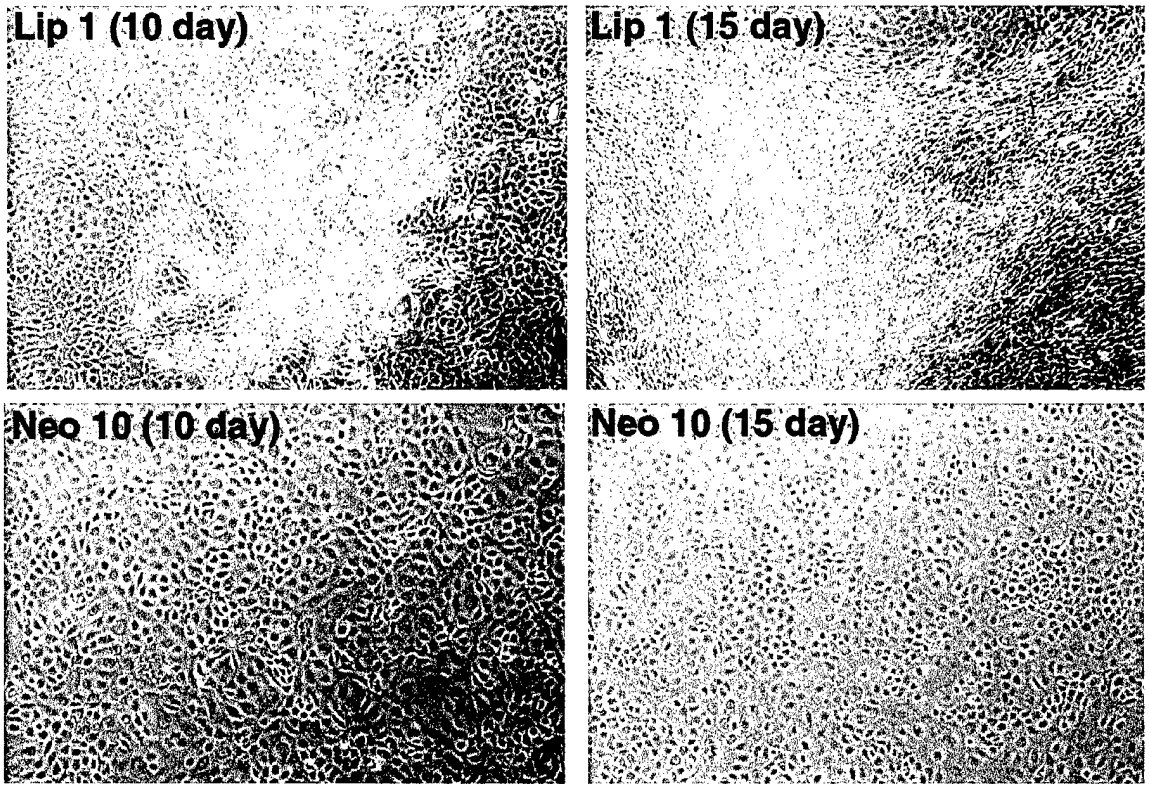

Fold Change in Growth
(MTS Units)
40
35
30
25
20
15
10
5
0
Neo
Lip
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days in Culture

Figure 1

A.


Lip 1 (10 day)
Lip 1 (15 day)
Neo 10 (10 day)
Neo 10 (15 day)

B.

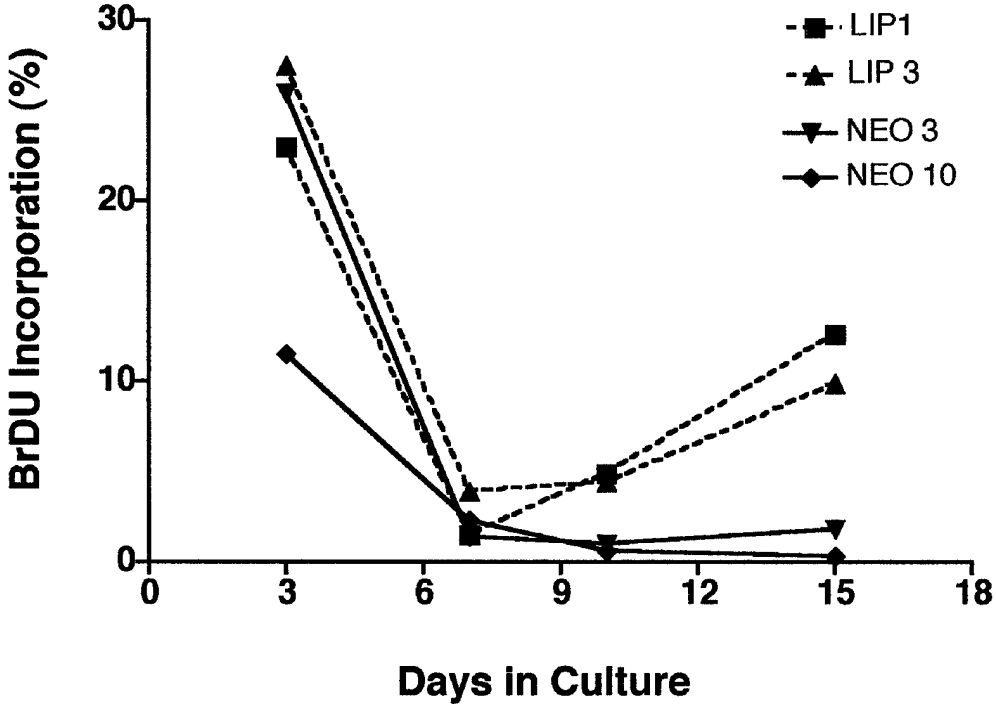
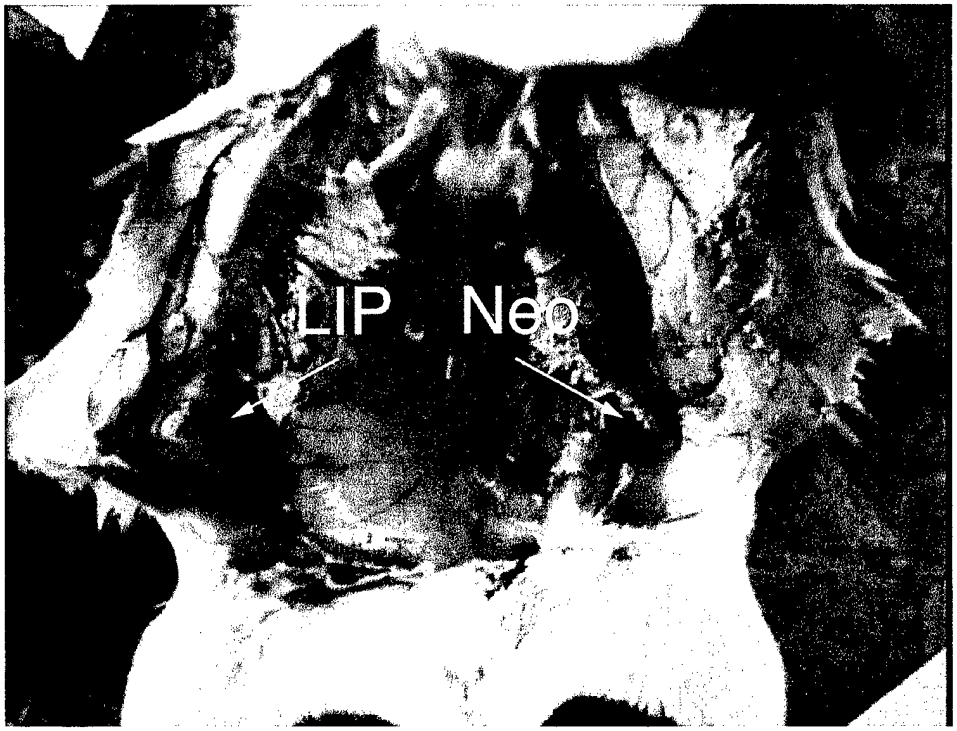

BrDU Incorporation (%)
30
20
10
0
0
3
6
9
12
15
18
Days in Culture
LIP1
LIP 3
NEO 3
NEO 10

Figure 2

A.


LIP
Neo

B.

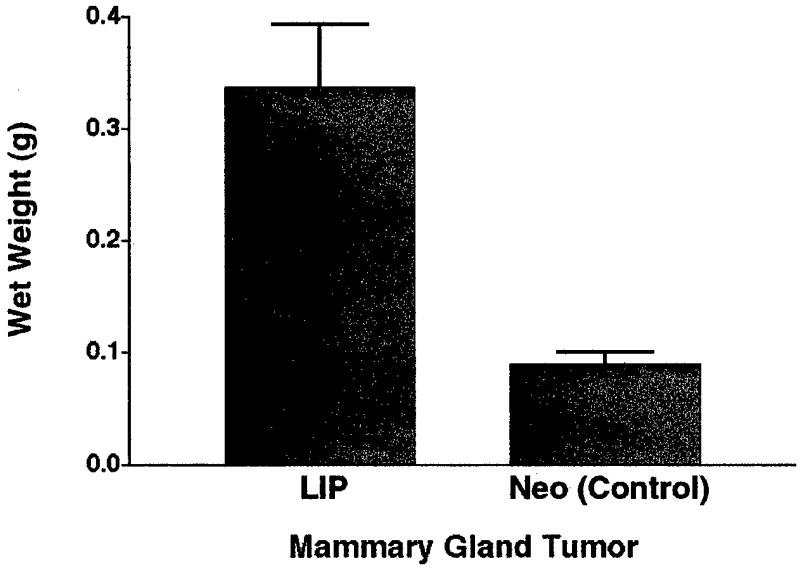

Wet Weight (g)
0.4
0.3
0.2
0.1
0.0
LIP
Neo (Control)
Mammary Gland Tumor

Figure 3

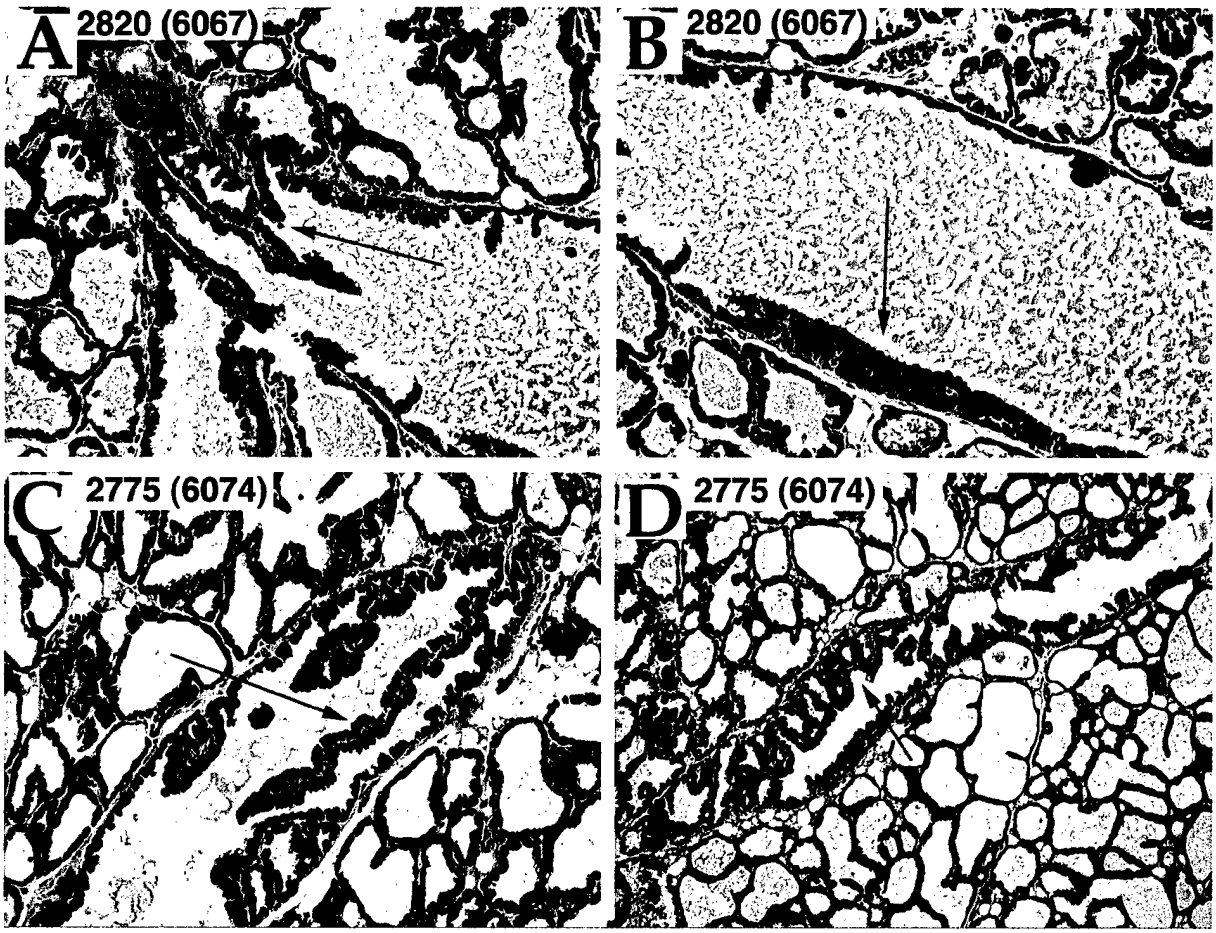


Figure 4

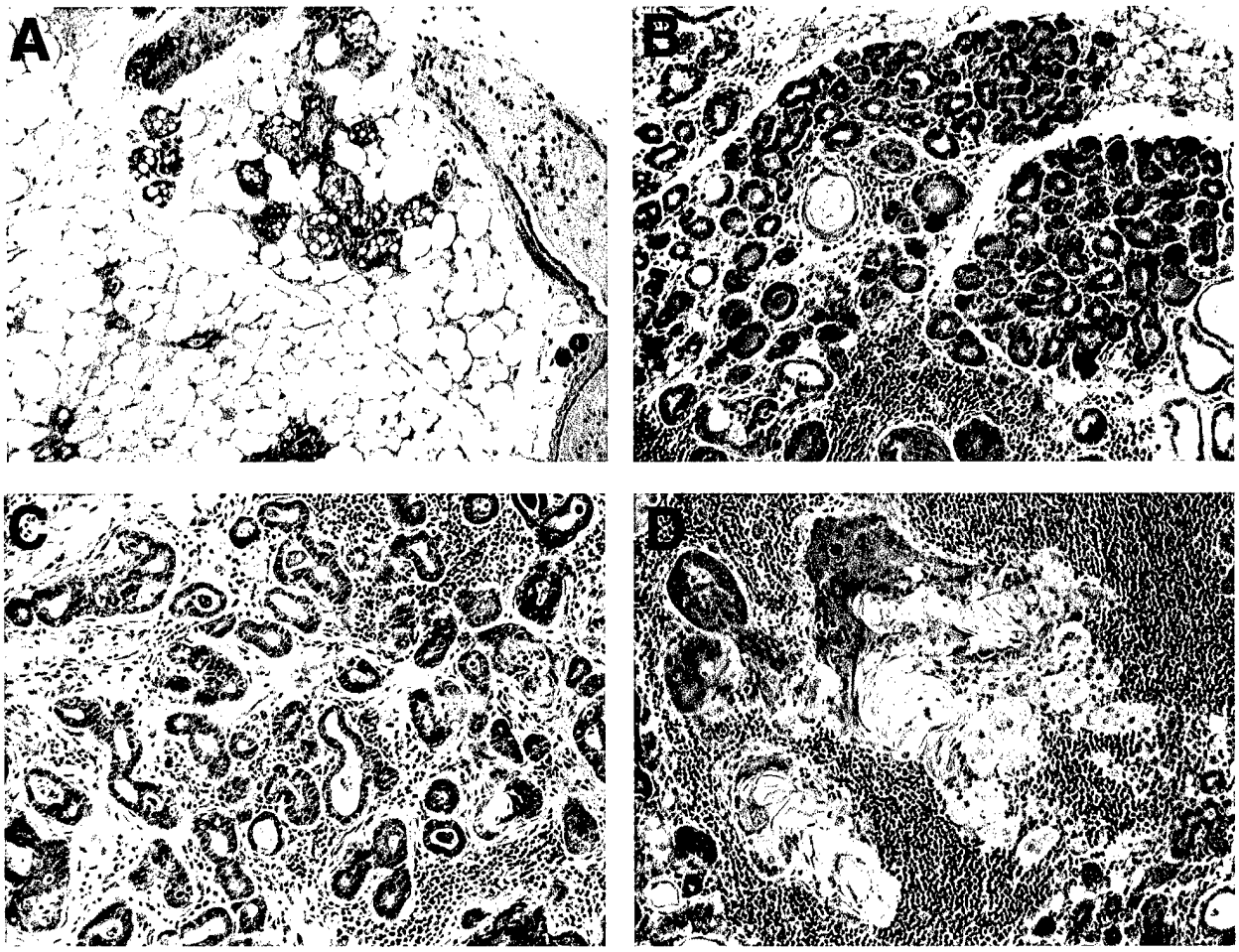


Figure 5

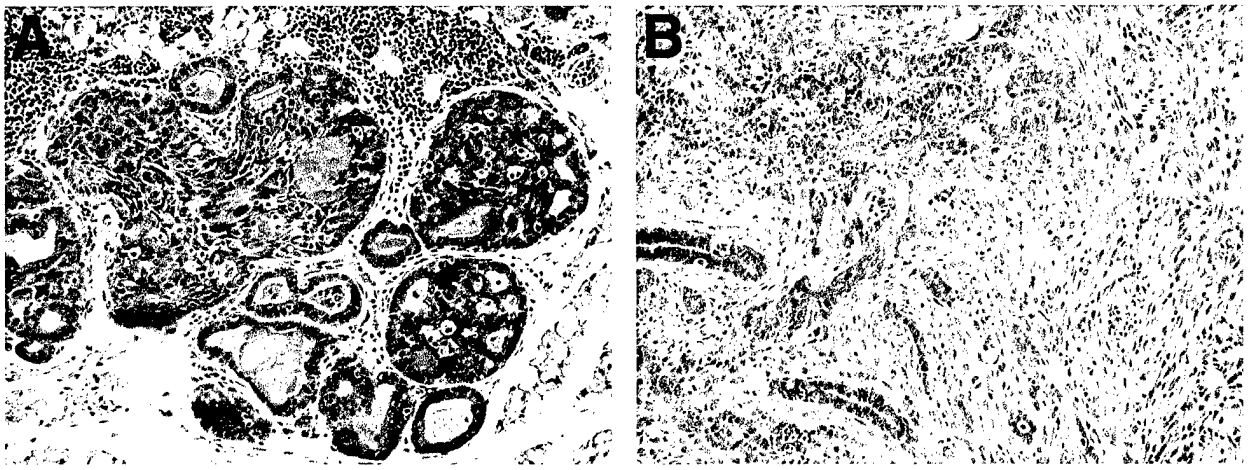


Figure 6

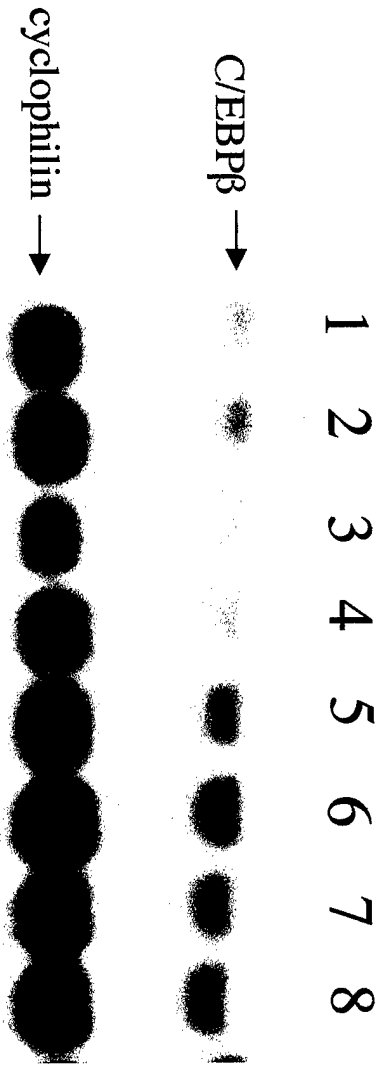


Figure 7

APPENDIX B (Manuscripts/Abstracts)

Biochem. Soc. Symp. **63**, 101–113
Printed in Great Britain

9

Composite response elements mediate hormonal and developmental regulation of milk protein gene expression

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Abstract

Our laboratory has been studying the mechanisms by which hormones regulate the expression of differentiated function in the normal mammary gland and how these regulatory mechanisms have deviated in breast cancer. Two rat milk protein genes, encoding β-casein and whey acidic protein, have been employed as molecular markers of mammary epithelial cell differentiation. Composite response elements containing multiple binding sites for several transcription factors mediate the hormonal and developmental regulation of milk protein gene expression. In the whey protein gene promoters, these include binding sites for nuclear factor (NF)-I, as well as the glucocorticoid receptor (GR) and signal transducers and activators of transcription (Stat5). In the casein promoters, these include binding sites for Stat5, Yin Yang 1 (YY1), GR and the CCAAT/enhancer binding protein (C/EBP). The C/EBP family of DNA binding proteins may play a pivotal role in maintaining the balance between cell proliferation and terminal differentiation in mammary epithelial cells. During normal mammary gland development, expression of LIP (liver-enriched inhibitory protein, a dominant-negative isoform of C/EBPβ) is hormonally regulated and correlates with cell proliferation during pregnancy. LIP can form heterodimers with other C/EBP family members and suppress their transcriptional activity. In contrast, C/EBPα is predominantly expressed during lactation following terminal differentiation. Elevated LIP levels have been detected in mouse, rat and human breast tumours of different aetiologies. This provides a mechanism, therefore, to block terminal differentiation and facilitate continued proliferation.

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Introduction

An understanding of the mechanisms regulating the development of the normal mammary gland is required if we are to fully understand the aberrant regulatory mechanisms responsible for breast cancer. Previous studies in our laboratory have been directed at defining the mechanisms by which hormones regulate lactation; specifically, how peptide and steroid hormones act in a synergistic manner to regulate the expression of milk protein genes. Recent studies from several laboratories, including our own, have led to the identification of the important elements required for mammary-specific gene expression, and have provided new insights into the mechanism of synergy of prolactin and glucocorticoids in regulating milk protein gene expression (reviewed in [1]).

Composite response elements have been identified which have a modular structure that is conserved in most mammals. These elements are sometimes duplicated in the 5′ flanking regions of the milk protein genes. In the casein promoters, these include binding sites for signal transducers and activators of transcription (Stat)5, Yin Yang 1 (YY1), CCAAT/enhancer binding protein (C/EBP) and the glucocorticoid receptor (GR) [2]. In the whey acidic protein (WAP) gene promoters, these include binding sites for nuclear factor (NF)-I, as well as the GR and Stat5 [3]. Thus tissue-specific expression does not appear to be mediated by the presence of a single factor, but instead requires co-operative interactions between several factors. These are mediated by both protein–DNA and protein–protein interactions. For example, direct protein–protein interactions have been demonstrated between the GR and both C/EBPα [4] and Stat5 (B. Groner, personal communication). Signal transduction pathways regulated by both peptide and steroid hormones play a critical role in transcription factor activation. Appropriate cell-specific regulation appears to require the interaction of both activators and repressors.

The following models have been proposed to explain the different kinetics of activation and the synergistic effects of glucocorticoids and prolactin on β-casein and WAP gene expression. For β-casein, glucocorticoids alter the ratio of the C/EBPβ liver-enriched inhibitory protein (LIP) and liver-enriched activating protein (LAP) isoforms [2], a slow process that requires ongoing protein synthesis. In contrast, prolactin rapidly activates Janus kinase 2 (JAK2) and Stat5 tyrosine phosphorylation, resulting in nuclear translocation and DNA binding. Direct C/EBP–GR and possibly Stat5–GR interactions facilitate the displacement of YY1 and hence transcriptional activation (Fig. 1).

For WAP, glucocorticoids rapidly induce changes in chromatin structure at the distal enhancer, facilitating the binding of NF-I [5]. Prolactin activation of Stat5 leads to a further enhancement of gene expression, and may again involve the direct interaction of Stat5 with the GR. These simplified models do not take into account the fact that additional positive (such as Ets/WAP and Oct-1/β-casein) and negative (single-stranded DNA binding proteins/β-casein) factors are also involved in the transcriptional regulation of these genes (reviewed in [6]). While less is known concerning the specific targets for insulin regulation of casein gene expression, this is most likely mediated through

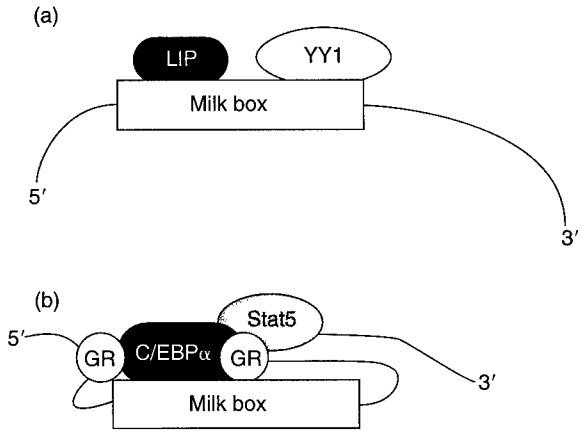

Fig. 1. Hormonal activation of casein gene expression. (a) A simplified model of the β-casein promoter 'milk box' region and its interaction with the transcription factors LIP and YY1, which repress transcription. (b) Activation of the β-casein promoter by glucocorticoids and prolactin results in a glucocorticoid-dependent switch in the C/EBPβ isoforms from LIP to LAP, followed by the induction of C/EBPα during lactation. Prolactin activation of Stat5 results in protein–DNA as well as protein–protein interactions between C/EBPα and the GR, between Stat5 and GR and possibly between C/EBPα and Stat5, leading to displacement of YY1 and gene activation.

insulin receptor substrate (IRS)-1 and IRS-2 protein scaffolding [7]. Cytokine activation of IRS-1/2 can also occur via JAK kinase activation [7].

Our recent studies have been focused primarily on investigating the regulation of three of these transcription factors and their isoforms, NF-I, Stat5 and C/EBP, during normal mammary gland development and in breast cancer. These will be summarized briefly below.

NF-I

In order to localize regulatory regions important for the hormonal and tissue-specific expression of the rat WAP gene, DNase I-hypersensitive sites were mapped in the 5′ flanking region of the rat WAP transgene that demonstrated copy-number-dependent expression in transgenic mice [3]. Two mammary-specific DNase I-hypersensitive sites were identified in lactating mice, and the region containing the distal site between 830 and 720 bp 5′ to the transcription start site was shown to be critical for expression in transgenic mice. Detailed analysis of this region, including genomic and *in vitro* DNase I and footprinting and dimethyl sulphate interference analyses, indicated that it contains several binding sites for the transcription factor NF-I [3].

The NF-I gene family consists of four highly related genes in mammals [8]. While the N-terminal DNA binding and dimerization domain is well conserved between all NF-I proteins, the C-terminal sequences diverge sub-

stantially. Alternative splicing leads to alterations in the C-terminal domain, possibly affecting the regulatory properties of different NF-I isoforms. At least 12 different NF-I isoforms have been identified, and further diversity exists because of the possibility of heterodimerization. Some of these NF-I isoforms contain a carboxyproline-rich *trans*-activation domain [8]. This *trans*-activation domain has considerable identity with the repeated C-terminal domain in RNA polymerase II [9].

There are now several examples of tissue-specific NF-I isoforms, and of co-operative interaction of these isoforms with nuclear hormone receptors in composite response elements, e.g. in the c-*fos* vitamin D response element [10] and the mouse mammary tumour virus long terminal repeat [11]. Both NF-I and C/EBPs are often found in composite response elements [12]. It has been suggested that NF-I might act to tether nuclear receptors via a combination of protein–protein and protein–DNA interactions. NF-I DNA binding activity has been shown to be responsive to cell–substratum interactions in mammary epithelial cells. Unlike with Stat5, this regulation appears to be independent of lactogenic hormones [13]. NF-I has also been shown recently to interfere with transformation induced by a number of different nuclear oncogenes [14] and to increase cell adhesion in chick embryo fibroblasts.

Surrounding the NF-I binding sites in the WAP distal promoter region, several specific GR binding sites have also been identified using *in vitro* DNase I footprinting with baculovirus-expressed GR [15]. This region was able to confer dexamethasone inducibility to a heterologous reporter gene in transient co-transfection experiments with GR in CVI cells [15]. Furthermore, glucocorticoid-induced changes in transgene expression were correlated with the appearance of DNase I-hypersensitive sites. Immediately downstream from the GR and NF-I binding sites is a consensus Stat5 binding site similar to that identified in the β-casein promoter.

To determine the functional importance of these sites, point mutations were introduced into the NF-I and mammary-gland-specific factor (MGF)/Stat5 binding sites and several independent lines of transgenic mice were analysed [5]. Transgene expression was totally abrogated when the palindromic NF-I site or both NF-I binding sites were mutated, and mutation of the MGF/Stat5 binding site reduced transgene expression by approx. 90% per gene copy. These results indicated that the regulation of WAP gene expression is determined by co-operative interactions among several transcription factors whose binding sites comprise a composite response element, and that NF-I plays a critical role in the regulation of WAP gene expression.

Gel-mobility-shift experiments have also suggested the existence of mammary-specific NF-I isoforms binding to the composite sites in the WAP distal enhancer [3]. Using reverse transcription–PCR and two consensus NF-I oligonucleotides, we have cloned a 1.6 kb cDNA from RNA isolated from the mammary gland of rats at day 10 of lactation (S.-J. Chen and J.M. Rosen, unpublished work). This cDNA clone is currently being used to screen a cDNA library prepared from the same RNA to identify any mammary-specific NF-I isoforms.

Stat5

Studies of the prolactin regulation of milk protein gene expression have led to the identification of a new member of the Stat family of transcription factors, originally designated as MGF but now known as Stat5, a ubiquitous factor that plays a critical role in cytokine regulation in a variety of different tissues and cell types [6,16,17]. Stat5 has now been shown to be a target for at least a dozen cytokines and several receptor tyrosine kinases, such as that for epidermal growth factor. Prolactin regulation of milk protein gene expression occurs in part through activation of the recently defined JAK/Stat pathway [6]. Prolactin receptor signalling can also be mediated through a number of different signalling pathways, including the activation of the mitogen-activated protein (MAP) kinase and Src pathways [18,19]. The JAK2 non-receptor tyrosine kinase has been demonstrated to be essential for prolactin activation of β-casein gene expression [20]. There is also recent evidence that autocrine and paracrine effects of prolactin may play important roles in regulating the growth of both rodent and human breast cancer cells [21–24]. Some of these effects of prolactin are mediated by activation of the JAK/Stat pathway.

The β-casein promoter contains the principal Stat5 response element that is now employed by many workers interested in cytokine action. Two different Stat5 genes, 5a and 5b, have been identified. The Stat5a and Stat5b gene products have similar but non-identical tissue distributions [25–27]. Both Stat5 isoforms are regulated by prolactin and many other cytokines, but functional differences between Stat5a and Stat5b remain to be established. In addition, alternatively spliced forms for both Stat5a and Stat5b have been described, both of which generate C-terminally truncated proteins that appear to function as dominant-negative isoforms [26,27]. The role of these different splice forms in normal mammary gland development and breast cancer has yet to be determined. However, elevated levels of members of the Stat family have been reported in nuclear extracts of breast carcinomas [28]. It is conceivable that both ligand-dependent and -independent mechanisms of Stat5 activation may, therefore, play an important role in breast cancer.

Two rat Stat5a isoforms (Stat5a1 and Stat5a2) resulting from alternative splicing were cloned and characterized in our laboratory [26]. A closely related rat Stat5b cDNA has also been isolated in collaboration with Dr. Li Yu-Lee at Baylor, and an alternatively spliced isoform of Stat5b, designated Stat5bΔ40C, containing a C-terminal truncation similar to that observed in Stat5a2, has been reported [27] (Fig. 2). Preliminary Northern blot (A. Kazansky and J.M. Rosen, unpublished work) and electrophoretic mobility shift assay (W. Doppler, personal communication) experiments have suggested that the levels of these alternatively spliced Stat5 isoforms may change during the transition from late pregnancy to early lactation. Co-transfection studies in COS cells have also indicated that these C-terminally truncated Stat5 isoforms display increased DNA binding to the β-casein γ-interferon-activated sequence (GAS) site, possibly due to a decreased dissociation rate. In addition, these C-terminally truncated isoforms display markedly reduced transactivation of β-casein

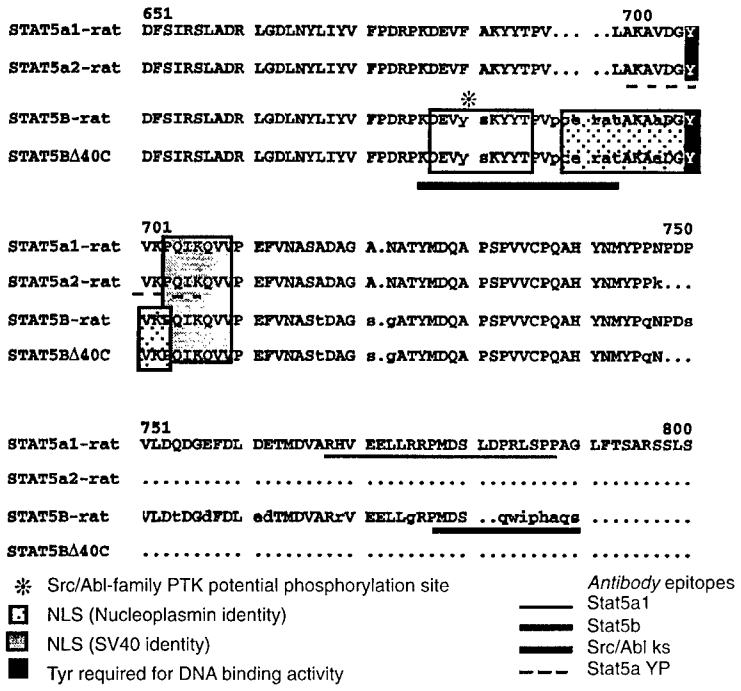

Fig. 2. Putative different functional domains in rat Stat5a and Stat5b. Sequences in the C-terminal regions of rat Stat5a and Stat5b are shown that may be responsible for differences in their nuclear localization and activation. The location of the C-terminal truncations present in the alternatively spliced isoforms of Stat5a and Stat5b is also shown. The underlined regions represent peptide epitopes used to generate specific antisera. Abbreviations: PTK, protein tyrosine kinase; NLS, nuclear localization sequence; SV40, simian virus 40.

promoter–reporter constructs (S. Lindsey and J.M. Rosen, unpublished work), and thus may be acting as dominant-negative isoforms.

A specific polyclonal antiserum to the C-terminal region of Stat5a1 was generated, and has been employed to study prolactin regulation not only in mammary epithelial cells but also in NB2 T-cells [29] and ovarian granulosa cells [30] in collaboration with Dr. Li Yu-Lee and Dr. JoAnne Richards at Baylor. This specific antibody was used to study Stat5a1 regulation during mammary gland development and its induction by prolactin in COS cells, and for immunohistochemical localization of Stat5a1. These studies indicated that Stat5a1 was expressed throughout mammary gland development, and that its expression was not correlated with maximal casein gene expression. Furthermore, studies on the activation of Stat5a1 in COS and HC11 cells have demonstrated a rapid and transient activation by prolactin that is not correlated with the long-term activation of casein gene expression.

Recently, we have also generated polyclonal antibodies that recognize the specific peptide containing the phosphotyrosine (equivalent to Tyr-694 in

sheep Stat5) required for Stat5 dimer formation and DNA binding, as well as antibodies to a unique sequence in Stat5b that we suggest may be a potential *src/abl* kinase site (Fig. 2). We hypothesize that this latter region may be an important determinant of the difference in the regulation and function of Stat5a and Stat5b. Using these antibodies, we have observed a difference in the kinetics of tyrosine phosphorylation of Stat5a and Stat5b in preliminary co-transfection experiments performed in COS cells (A. Kazansky and J.M. Rosen, unpublished work). Thus Stat5a is maximally tyrosine phosphorylated within 30 min following prolactin addition, decreasing at 4.5 h and to a much greater extent at 13.5 h, while Stat5b increases in its PY-20 and *src/abl* kinase site antibody reactivity during this period. Phosphorylation on additional tyrosine(s) of Stat5b appears to occur during these later times. Thus the phosphorylation status of the two Stat5 isoforms is different, possibly reflecting different kinase as well as phosphatase susceptibilities. It is conceivable that the differential activation of Stat5b compared with Stat5a may result in differential gene activation, giving rise to a proliferative or a terminally differentiated state respectively.

C/EBP

The C/EBPs are members of the leucine zipper class of sequence-specific, bZIP DNA binding proteins [31]. Several C/EBP family members (C/EBPα, β, δ and γ) have been described that are encoded by separate, intron-free genes and which share a conserved C-terminal DNA binding domain and basic leucine zipper dimerization domain, but differ in their N-terminal *trans*-activation domains [32]. The gene encoding C/EBPβ is transcribed into a single mRNA. Translation of this mRNA occurs from three different in-frame AUG codons via a leaky ribosome scanning mechanism, resulting in the synthesis of three proteins: two LAPs (LAP1, 39 kDa; LAP2, 36 kDa) and LIP (20 kDa) (Fig. 3) [33]. As with all C/EBP family members, LAP and LIP are capable of forming homo- or hetero-dimers with each other. LAP and LIP share the same DNA binding and dimerization domains, but LIP lacks the *trans*-activating N-terminus, rendering it able to antagonize the transcriptional activating potential of LAP by competing for the DNA binding site or by its interaction in a LAP/LIP heterodimer [33]. Thus the LAP/LIP ratio may be an important indicator of C/EBPβ transcriptional activity [33].

The LAP/LIP ratio is most likely regulated at the translational level by the mRNA 5′ cap binding protein [i.e. the eukaryotic translation initiation factor 4E (eIF4E)]. Thus the C/EBPβ mRNA provides a unique system with which to study eukaryotic translational control. RNAs encoding proteins involved in cell cycle progression, such as growth factors, oncogenes and *trans*-acting factors, possess long 5′ untranslated regions with extensive secondary structure [34]. The C/EBPβ mRNA is one such molecule, and is, therefore, translated inefficiently. Initiation is the rate-limiting step in translation, and eIF4E is present at limiting concentrations in the cell [35]. In quiescent cells, much of the eIF4E pool is complexed with one of a family of cytoplasmic inhibitors, the eIF4E binding proteins [36]. Mitogenic stimulation leads to

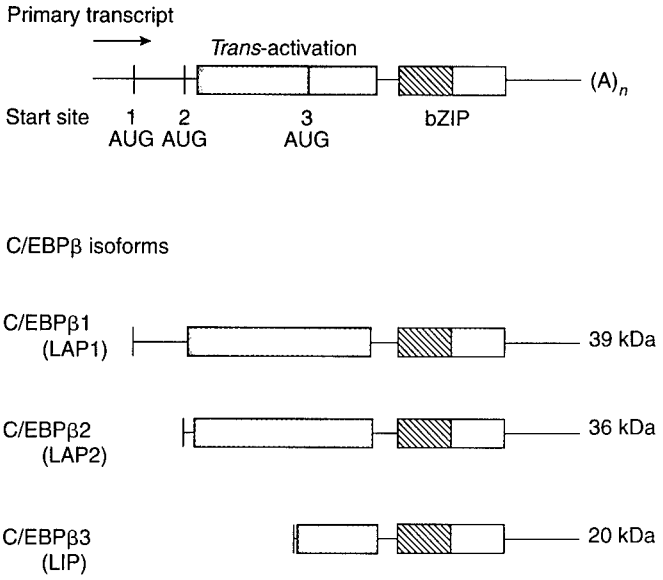

Fig. 3. Leaky ribosome scanning on a single mRNA generates three C/EBPβ isoforms. Diagrammatic representation of C/EBPβ mRNA with its three potential translation start sites (AUG). Sites number 1, 2 and 3 have relationships with the Kozak consensus sequences of 5/9, 6/9 and 9/9 respectively. The location of the *trans*-activation and DNA binding and dimerization domains (bZIP) are shown. Adapted from [53].

phosphorylation of eIF4E and of its binding proteins, which disrupts these complexes and increases the pool of active eIF4E. Increased eIF4E enhances the translation rate of inefficiently translated mRNAs [37].

RNA secondary structure causes significant ribosome stalling, so in unstimulated cells the few ribosomes that scan through to the C/EBPβ translation start sites are predicted to recognize the first two (at nucleotides 439 and 502) much more frequently than the third (at position 895). Thus in unstimulated cells the LAPs are predicted to be synthesized at a constitutive low level, and little LIP would be produced. In mitogenically stimulated cells, containing a much larger eIF4E pool, many more ribosomes should scan the C/EBPβ mRNA. The secondary structure of the C/EBPβ mRNA should thus be decreased significantly, and many more ribosomes should scan through the 5′ untranslated region. The LAP translation start sites are weak (5/9 and 6/9 nt match) as compared with the Kozak consensus sequence. However, the LIP start site conforms perfectly to this consensus [38]. Ribosomes unencumbered by secondary structure may often skip the imperfect Kozak sequences and recognize the LIP start site. Therefore the LAP/LIP ratio is predicted to be much lower in stimulated cells.

The C/EBP family is involved in the transcriptional regulation of genes important in the differentiation of many cell types, including myocytes, myelomonocytes, hepatocytes, adipocytes, ovarian follicles, intestinal epithe-

lium and mammary epithelial cells [2,39,40]. C/EBP transcriptional activity can be modulated by the level of expression of the various C/EBP proteins and their respective isoforms. Differences in dimerization ratios due to an abundance or lack of a specific isoform may alter C/EBP DNA binding affinities or *trans*-activating potential.

C/EBPα, β, and δ expression is differentially regulated throughout the development of several different tissues. A regulated pattern of C/EBP isoform expression occurs during mouse 3T3-L1 cell differentiation. C/EBPβ and C/EBPδ are expressed during the early phase of 3T3-L1 differentiation into adipocytes. This expression declines and is replaced by high levels of C/EBPα as the cells progress towards terminal differentiation [32]. This temporal pattern of expression supports the hypothesis that C/EBPβ and C/EBPδ can induce C/EBPα expression, which then contributes to terminal cell differentiation by arresting adipocyte proliferation [32,41]. The inhibition of cell proliferation by C/EBPα occurs in many cell types, and does not appear to require the presence of p53 or retinoblastoma protein (Rb) [42]. Overexpression of dominant-negative isoforms of C/EBP, such as LIP [40] or CHOP/GADD153 (growth arrest and DNA damage inducible gene 153) [43], will prevent adipogenesis.

Cellular proliferation of HepG2 hepatoma cells is also blocked by LAP [44]. LAP arrests the cell cycle before the G1/S boundary in hepatoma cells, and this effect can be antagonized by LIP. During rat postnatal development, the levels of LAP in liver nuclei rise much more than those of LIP [33]. It has been suggested that the LAP/LIP ratio is more important for the regulation of gene expression than are the levels of LAP alone [33]. If so, perhaps LIP levels modulate the effect of LAP on the cell cycle and LIP expression in the hepatoma cells is not sufficient to antagonize LAP's inhibition of proliferation.

C/EBPβ activity is regulated not only by changes in expression level but also by post-translational modifications. The cell type and developmental specificity of C/EBPβ-regulated gene expression and *trans*-activation appears to be a result of interactions with multiple signal transduction pathways. Activation of these pathways by external stimuli can lead to phosphorylation and resultant increases in the *trans*-activating potential of C/EBPβ. Several protein kinases, including Ca^{2+}/calmodulin-dependent protein kinase [45], protein kinase C [46] and MAP kinase [47], have been shown to phosphorylate Ser/Thr residues at several different positions, resulting in increased C/EBPβ activity.

Recently, it has been suggested that C/EBPβ contains two conserved regions which can interact with both the *trans*-activation and DNA binding domains to repress DNA binding and transcriptional activity [48,49]. These inhibitory regions are positioned between the activation and DNA binding domains (Fig. 4). Studies have demonstrated that phosphorylation within an inhibitory region prevents intramolecular interaction with these domains and permits C/EBPβ *trans*-activation. Several phosphorylation sites are located within these inhibitory regions and may contribute to C/EBPβ *trans*-activation through modification of protein conformation [49]. Interestingly, it has also been demonstrated that these inhibitory regions are capable of forming intermolecular interactions with each other [49]. Examination of these inter-

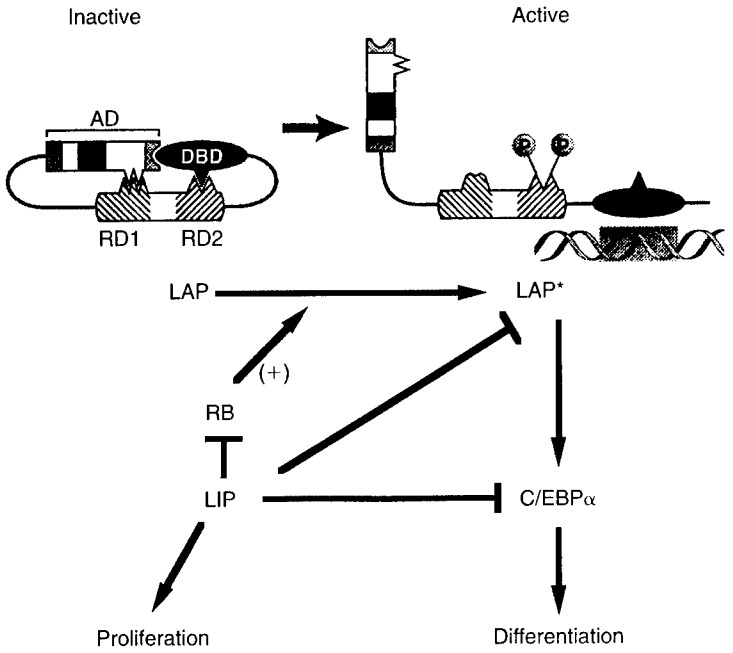

Fig. 4. LIP expression can influence hormonally regulated proliferation or terminal differentiation. The transient interaction of the hypophosphorylated form of Rb with LAP results in a conformational change in LAP similar to that observed following increased phosphorylation (adapted from [49]). This can lead to increased transcriptional activation of C/EBPβ-responsive genes, including C/EBPα, resulting in withdrawal from the cell cycle and terminal differentiation. Leaky ribosome scanning in breast cancer cells results in increased translation of the LIP isoform of C/EBPβ. This dominant-negative isoform can inhibit both LAP and C/EBPα, as well as interacting directly with Rb, preventing terminal differentiation and favouring proliferation. Abbreviations: AD, *trans*-activation domain; DBD, DNA binding domain; RD, regulatory domain.

actions may provide valuable information to further our understanding of LIP's antagonistic effect on the transcriptional activity of C/EBP family members.

Traditionally, C/EBP family members have been described as DNA binding proteins which regulate the transcription of genes involved in cellular differentiation. It has now become apparent that C/EBPβ is additionally able to interact with proteins involved in cell cycle control. Rb, a regulator of cell cycle progression, is important for cellular differentiation and tumour suppression. During G0 and G1 of the cell cycle, hypophosphorylated Rb prevents cell growth by blocking progression through the cell cycle. Hyperphosphorylation of Rb during mid-G1 permits cell growth and induces proliferation. All three C/EBPβ isoforms have been observed to interact directly with the simian virus 40 T antigen domain of hypophosphorylated Rb in differentiating or differentiated cells [50]. Two regions of C/EBPβ have been identified to interact with

Rb. One of these regions, in the N-terminus, is found in both LAP isoforms and is 54% similar to the sequence used by the transcription factor, E2F-1, to interact with Rb [50]. An additional binding region is located in the C-terminus and is present in the LIP and LAP isoforms. Transient interaction with Rb increases the DNA binding activity and *trans*-activation potential of the C/EBPβ isoforms, which may in turn activate the transcription of genes involved in cellular differentiation, such as C/EBPα [50].

Elevations in LIP expression should interfere with the activation of LAP by Rb and may antagonize the ability of LAP to transcriptionally regulate genes involved in cellular differentiation. The net result of increased LIP levels might, therefore, include an inhibition of cellular differentiation resulting in excessive proliferation and tumour growth. We propose, therefore, that the interaction of the C/EBPβ LAP isoforms with Rb is a switch to promote cellular differentiation, and that this may be blocked by increased LIP expression in breast cancer (Fig. 4). A decrease in the expression of C/EBPα, as well as rearrangements in the genes for the various C/EBP family members, has also been associated with tumour development in a number of other cell types [51,52].

Coupled with recent observations on the interaction of the hypophosphorylated form of Rb with C/EBPβ, this has led us to propose a hypothesis to explain why hormones that are responsible for hormone-dependent terminal differentiation in the normal mammary gland may elicit hormone-dependent growth in some breast cancers (Fig. 4). Thus, in breast cancer, LIP expression may be elevated as a function of leaky ribosome scanning. This most probably results from altered phosphorylation of translation initiation factors as a consequence of growth factor or oncogene activation of signal transduction pathways in breast cancer cells. At the same time overexpression of eIF4E, which has also been reported to increase levels of cyclin D1, may result in increased proliferation [37]. LIP expression will result in heterodimer formation with LAP (and C/EBPα), inhibit C/EBPα induction and prevent terminal differentiation (Fig. 4). This provides a mechanism to block terminal differentiation and facilitate continued proliferation. This model is consistent with the observation that LIP expression is detected in breast tumours, especially more aggressive tumours that are oestrogen- and progesterone-receptor-negative (C. Zahnow, B. Raught, A. James and J.M. Rosen, unpublished work).

The preceding studies are consistent with the general hypothesis that aberrant hormonal regulation of specific transcription factor isoforms can result in continued proliferation and failure to undergo terminal differentiation and apoptosis of breast epithelial cells. An understanding of the mechanisms regulating the expression and function of these different isoforms in normal mammary gland development will, therefore, be important for the understanding the aetiology of, and developing new treatments for, breast cancer.

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Overexpression of C/EBPβ-LIP, a Naturally Occurring, Dominant-Negative Transcription Factor, in Human Breast Cancer

*Cynthia A. Zahnow, Pamela Younes, Rodolfo Laucirica, Jeffrey M. Rosen**

Background: **When cells fail to maintain a balance between proliferation, terminal differentiation, and programmed cell death, cancer often results. The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors regulates many genes involved in the processes of proliferation and terminal differentiation. The messenger RNA for C/EBPβ is translated into two major isoforms, LAP (liver-enriched activating protein) and LIP (liver-enriched inhibitory protein). LIP levels appear to be elevated in mouse mammary tumors but not in hyperplastic mammary tissues. We tested whether LIP expression is elevated in human breast cancer and whether elevated expression is associated with biologic predictors of the aggressiveness of the disease.** *Methods:* **Homogenates of infiltrating ductal carcinoma specimens from 39 women were analyzed for C/EBPβ protein content by western blot analysis, and the ratio of LAP to LIP in specimens containing high levels of LIP (i.e., levels approximately 15 times higher than those in tumor specimens that express little or no LIP) was also determined. Nonparametric statistical analyses were performed.** *Results:* **LIP was present at high levels in nine of 39 specimens of infiltrating ductal carcinoma. Eight of the nine specimens of infiltrating ductal carcinoma that contained high levels of LIP were negative for estrogen receptor and progesterone receptor (ER^-/PR^-); all nine tumors were aneuploid and poorly differentiated, and eight of nine were highly proliferative. Of the tumors that contained LIP at low or nondetectable levels, six of 30 were ER^-/PR^-, 17 of 29 were aneuploid, eight of 27 were highly proliferative, and 11 of 30 were poorly differentiated.** *Implication:* **LIP expression should be evaluated further as a prognostic marker for patients with breast cancer.** **[J Natl Cancer Inst 1997;89:1887–91]**

Tumorigenesis often results from the failure of cells to maintain a balance between proliferation, terminal differentiation, and programmed cell death. The CCAAT/enhancer-binding protein (C/EBP) family of basic leucine zipper (bZIP), DNA-binding proteins may play a pivotal role in maintaining this balance by regulating the expression of genes involved in proliferation and terminal differentiation *(1–3)*. Currently, the genes for six C/EBPs (C/EBPα, β, δ, γ, ε, and ζ) have been characterized with the use of nomenclature introduced by Cao et al. *(3)*. With the exception of C/EBPε and ζ, all of the genes in this family are intronless and share a conserved carboxyl-terminal DNA-binding domain and bZIP dimerization domain but differ in their amino-terminal *trans*-activation domains *(4)*. The gene encoding C/EBPβ is transcribed into a single messenger RNA that is translated in the mammary gland by a leaky ribosome-scanning mechanism, resulting in the synthesis of two principal isoforms designated liver-enriched activating protein (LAP—35 kd) and liver-enriched inhibitory protein (LIP—20 kd). Consequently, studies of LAP/LIP gene regulation can be conducted only at the protein level. As with all C/EBP family members, LAP and LIP are capable of forming homodimers or heterodimers with each other as well as with other leucine zipper proteins. LIP displays an increased affinity for DNA, but it lacks a portion of its *trans*-activating domain, rendering it able to antagonize the transcriptional activation of LAP or other C/EBPs and leucine zipper proteins, in substoichiometric ratios *(5)*.

Recently, we have reported that C/EBPβ-LIP expression is elevated in transplantable and primary mouse mammary tumors of different etiologies but is not expressed in transplantable, preneoplastic mammary hyperplasias *(6)*. These data suggested that LIP expression may play a role in rodent mammary tumorigenesis, but its role in human breast cancer was undetermined. We hypothesized that an increase in the levels or activity of LIP may inhibit terminal differentiation and help facilitate uncontrolled proliferation and tumorigenesis that may result from other genetic alterations known to occur in breast cancer, e.g., amplification of growth factor receptors such as erbB2, increased expression of cyclin D1, and loss or mutation of p53. LIP is of particular interest because it represents a translationally regulated, naturally occurring, dominant-negative C/EBP family member that may play a role in breast cancer. Consequently, the purpose of this study was to determine if LIP expression is elevated in human breast cancer and whether this overexpression is associated with biologic predictors of aggressive behavior in human breast cancer, such as histologic and nuclear grade, cell proliferation, DNA ploidy, and estrogen receptor (ER) and progesterone receptor (PR) status.

Materials and Methods

Western Blot Analysis

Infiltrating ductal carcinoma specimens from 37 patients and normal breast tissue from 10 patients were provided by the Baylor College of Medicine Tissue Bank, Department of Pathology. Specimens of infiltrating ductal carcinoma from two patients were provided by the Providence Health Center, Waco, TX. Seven of the 10 normal tissues were paired controls from patients with infiltrating ductal carcinomas examined in this study, and the remaining three were age-matched controls. Normal tissue is defined as tissue resected greater than 2 cm from the margins of the tumor, containing minimal adipose tissue and a higher proportion of breast parenchyma. The use of discarded human tissue was approved by the Baylor Institutional Review Board. Tissues were disrupted in RIPA buffer—50 m*M* Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% desoxycholate, 150 m*M* NaCl, 1 m*M* EGTA [i.e., ethylene glycol-bis(β-aminoethyl ether)-*N,N,N,N'*-tetraacetic acid], and 0.2% sodium dodecyl sulfate (SDS)—containing the following kinase, phosphatase, and protease inhibitors: 1 m*M* $NaVO_3$, 1 m*M* NaF, 1 m*M* Na_2MoO_4, 10 n*M* okadaic acid, and 1 μg/mL benzamidine, aprotinin, soybean trypsin in-

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See "Notes" following "References."

hibitor, and antipain. Aliquots of these lysates containing 100 μg of protein were subjected to electrophoresis on denaturing SDS–12% polyacrylamide mini gels and then transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA) overnight at 75 mA. Blots were blocked 90 minutes in TBST (20 m*M* Tris [pH 7.5], 150 m*M* NaCl, and 0.5% Tween 20) containing 3% non-fat dry milk (Carnation, Glendale, CA). They were then incubated for 90 minutes in this solution containing antibodies (0.5 ng/mL) (Santa Cruz Biochemicals, Inc., Santa Cruz, CA) prepared against C/EBPβ. Blot washes consisted of TBST (without milk); the washing was done three times for 5–10 minutes each, with agitation. Blots were then incubated for 60 minutes in blocking solution containing 200 ng/mL biotinylated donkey anti-rabbit immunoglobulin (Amersham, Little Chalfont, U.K.) and then washed. Lastly, blots were incubated for 30 minutes in blocking solution containing 40 ng/mL streptavidin–horseradish peroxidase (Oncogene Science, Uniondale, NY) and washed as before. Enhanced chemiluminescence (Hyperfilm; Amersham) and chemifluorescence reagents (Storm Fluoroimager; Molecular Dynamics, Sunnyvale, CA) were used for visualization, as per the manufacturer's instructions.

To determine the sensitivity of western blot analysis for detection of C/EBPβ expression, we used both the Amersham enhanced-chemiluminescence (ECL) and the enhanced-chemifluorescence (ECF) detection systems to analyze increasing amounts of a C/EBPβ protein standard isolated from previously characterized mouse mammary tumors generated by overexpression of transforming growth factor-α (TGF-α) *(6)*. The ECL data were analyzed by use of multiple exposures of Hyperfilm and quantitated with Adobe Photoshop (Adobe Systems, Mountain View, CA), and the ECF data were analyzed by use of a STORM imaging system (Molecular Dynamics) and Image Quant software (Molecular Dynamics). Levels of LAP and LIP were linear over a larger range with the use of ECF detection (12.5–400 μg/lane) as compared with ECL detection (6.25–100 μg/lane) (data not shown). Consequently, for purposes of quantitation, each western blot was analyzed with the use of the ECF detection system, and levels of LAP and LIP were analyzed as "-fold" and/or percent change from the C/EBPβ protein standard (100 μg). Because a source of purified C/EBPβ protein was not available, an extract of TGF-α-induced mammary tumors (in RIPA buffer) that contained large amounts of the C/EBPβ isoforms was used as the standard. The values were plotted on a scatter diagram, and the data for LIP expression were separated into two distinguishable groups (data not shown). Standard values were assigned the value of 1 or 100%. High LIP values ranged from 5.2-fold above the standard to 0.5-fold below the standard. Likewise, lower LIP (low or nondetectable) values ranged from 0.3-fold below standard values to 0. None of the data points were positioned between the values of 0.53 (53%) and 0.25 (25%). Consequently, values that were greater than 50% below the value for the C/EBPβ protein standard were classified as high, and those that were less than 25% below the standard were classified as low or nondetectable. As a visual check for uniform gel loading, all blots were stained with the Reversible Protein Detection Kit (Sigma Chemical Co., St. Louis, MO).

Immunohistochemistry

Tissues were fixed overnight in 10% neutral buffered formalin, embedded, sectioned, deparaffinized through a graded series of xylenes and alcohols, and then rehydrated in water and phosphate-buffered saline. Antigens were retrieved by boiling the tissue sections for 10 minutes in 2 *M* urea, and endoperoxidases were blocked in a methanol solution containing 3% H_2O_2 for 15 minutes at room temperature. After being washed, tissue sections were incubated for 1 hour at room temperature with 10% normal goat serum (Sigma Chemical Co.) and 20% avidin blocking solution (Vector Laboratories, Inc., Burlingame, CA) in 1× PBST (i.e., phosphate-buffered saline containing 0.1% Tween 20). Excess blocking solution was drained, and polyclonal rabbit primary antibody for C/EBPβ (1:300; Santa Cruz Biochemicals, Inc.) containing 20% biotin blocking solution (Vector Laboratories, Inc.) in 10% normal goat serum was applied overnight at 4 °C. Biotinylated goat anti-rabbit secondary antibody at a 1:200 dilution in 10% normal goat serum was incubated for 30 minutes at room temperature. Tissue sections were then incubated for 30 minutes at room temperature with the preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain *Elite* ABC; Vector Laboratories, Inc.). Colorimetric detection by use of the chromagen diaminobenzidine tetrahydrochloride was carried out according to the manufacturer's instructions (Vector Laboratories, Inc.). Control slides were treated identically except that 6 μg of C/EBPβ peptide (Santa Cruz Biochemicals, Inc.) was incubated with the primary antibody for 15–30 minutes at 0 °C.

Analysis of Prognostic Indicators

Analyses of ER and PR status were performed at OncQuest, Specialty Laboratories, Inc., Santa Monica, CA, by use of a dextran–charcoal-binding assay. For consistency in histologic grading, all slides were examined by one pathologist (R. Laucirica), and the grade for each infiltrating ductal carcinoma was assessed by use of the method of Elston and Ellis *(7)*. DNA flow cytometry analysis of tumors procured at the time of surgery and MIB-1 (Immunotech, Inc., Westbrook, ME) immunohistochemistry, which detects the Ki67 nuclear antigen associated with cellular proliferation *(8)*, were conducted in the Department of Pathology, The Methodist Hospital. The DNA content (ploidy) for 37 of 39 cases was assessed by use of a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and Modfit software (Verity Software House, Topsham, ME). Nonparametric statistical analysis was performed in consultation with a biostatistician (Dr. Charles Miller, Department of Surgery, Baylor College of Medicine). *P* values were computed by Fisher's exact test (two-sided) and the Mann-Whitney *t* test (two-sided).

Results

LIP is an amino-truncated version of the C/EBPβ LAP isoform. Therefore, C/EBPβ antibodies, which recognize a carboxy-terminal epitope in C/EBPβ proteins, detect both LAP and LIP isoforms. Accordingly, the antibodies that recognize the amino-terminal region do not detect LIP. Consequently, no antibodies that can selectively distinguish LIP are currently available. Size fractionation of the C/EBPβ protein isoforms by SDS–polyacrylamide gel electrophoresis followed by western blotting with the use of an antibody to the carboxy-terminus of C/EBPβ, however, permitted the detection of the different C/EBPβ isoforms. At present, this is the only technique available for the analysis of LIP expression.

To address the question, "Is LIP expressed in human breast cancer?," we analyzed infiltrating ductal carcinomas from 39 women, aged 26–83 years old. As evidenced by western blot analyses, levels of LAP and LIP expression within the infiltrating ductal carcinomas were quite variable among the different patients (Fig. 1, A, B, and C). Additional protein bands, representing both cross-reactive proteins and phosphorylated or other post-translationally processed LAP and LIP isoforms, were also visible in many tumors.

For the determination of the significance of elevated levels of LIP expression in some tumors, an expression level was assigned (as described in the "Materials and Methods" section) to the LIP values and was tested for association with various prognostic factors. LIP was expressed at high levels in 23% (nine of 39) of the infiltrating ductal carcinomas. Examples of high levels of LIP expression are shown in Fig. 1, B (lanes 2, 3, and 6). LIP levels designated as "high" (mean value ± 95% confidence interval: 1.59 ± 0.6) were on average 15 times greater than the levels of LIP in the lower expressing (mean value ± 95% confidence interval: 0.107 ± 0.02) or nonexpressing tumors ($P<.0001$, two-sided Mann-Whitney *t* test). In "normal" breast tissue, LIP levels were low or nondetectable above background values.

Because the amino-truncated LIP isoform has a greater DNA affinity than the LAP isoform and can heterodimerize and inhibit the transactivation ability of other C/EBPs at a substoichiometric ratio as low as 1:5 (20%), the ratio of LIP to LAP was determined for the predominant LAP isoform (35 kd) that also has the greatest transactivation potential *(5)*. In the tumors expressing the highest LIP levels, the average ratio of LIP to LAP was determined

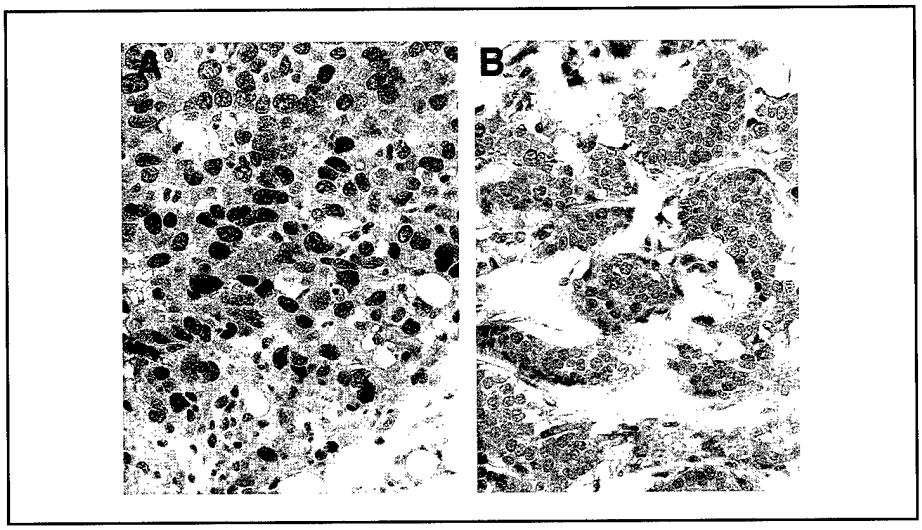

Fig. 2. Immunohistochemical localization of CCAAT/enhancer-binding protein β (C/EBPβ) isoform expression in a human Elston–Ellis grade III, estrogen receptor-negative and progesterone receptor-negative, infiltrating ductal carcinoma **(A)** and an Elston–Ellis grade I, estrogen receptor-positive and progesterone receptor-positive, infiltrating ductal carcinoma **(B).** Note the intense staining of pleomorphic nuclei in **panel A** and much weaker staining in **panel B.** In agreement with these results, western blot analysis of the infiltrating ductal carcinoma in **panel A** shows strong expression of the C/EBPβ isoforms (*see* Fig. 1, B, lane 6), but the infiltrating ductal carcinoma in **panel B** expresses the isoforms at a much lower level (*see* Fig. 1, A, lane 3). Viewed with a 40× objective.

B). Surrounding normal tissue also expressed C/EBPβ in both the epithelium and stroma (data not shown). The specificity of the staining was confirmed by selective competition with the peptide against which the carboxy-terminal antiserum was generated, but not by a nonspecific peptide (data not shown). These results from immunohistochemical analyses have confirmed that the C/EBPβ isoforms are expressed strongly in malignant epithelial cells of aggressive, poorly differentiated infiltrating ductal carcinomas (Fig. 2, A and B). However, development of a double fluorescent-imaging technique using amino- and carboxy-terminal antisera will be required to detect the selective localization of LIP expression in the future. Examination of hematoxylin–eosin-stained microscopic tissue sections of both the high and the low LIP-expressing tumors also demonstrated that many of the less aggressive, low LIP-expressing tumors possessed large sheets of tumor epithelium, as illustrated in Fig. 2. Consequently, differences in the ratio of LIP to LAP cannot be accounted for by variations in epithelial cell number.

Discussion

Breast cancer is thought to progress through multiple morphologic stages, beginning with typical hyperplasia and progressing through atypical hyperplasia, *in situ* carcinoma, invasive carcinoma, and eventual metastasis. Invasive carcinomas are also heterogeneous with respect to their proliferative and invasive potential. In this study, C/EBPβ protein isoforms and, in particular, the naturally occurring, dominant-negative LIP isoform have been detected and are more highly expressed in ER^-/PR^-, aneuploid, Elston–Ellis grade III infiltrating ductal carcinomas than in the less aggressive, ER^+/PR^+, diploid, Elston–Ellis grade I tumors. This observation is consistent with the hypothesis that the C/EBPβ isoforms may play a role in regulating terminal differentiation and cell cycle progression, as illustrated in Fig. 3.

Proliferative diseases such as cancer often result from failure to withdraw from the cell cycle at the G_1 checkpoint. Multiple signal transduction pathways, generated by diverse extracellular and intracellular factors, converge at this restriction point and influence cell cycle progression. This advancement beyond late G_1 is believed to be a result of the phosphorylation and consequent inactivation of the retinoblastoma protein (Rb). It has been demonstrated that Rb interacts directly with and activates all of the C/EBPβ isoforms *(10);* however, how this interaction

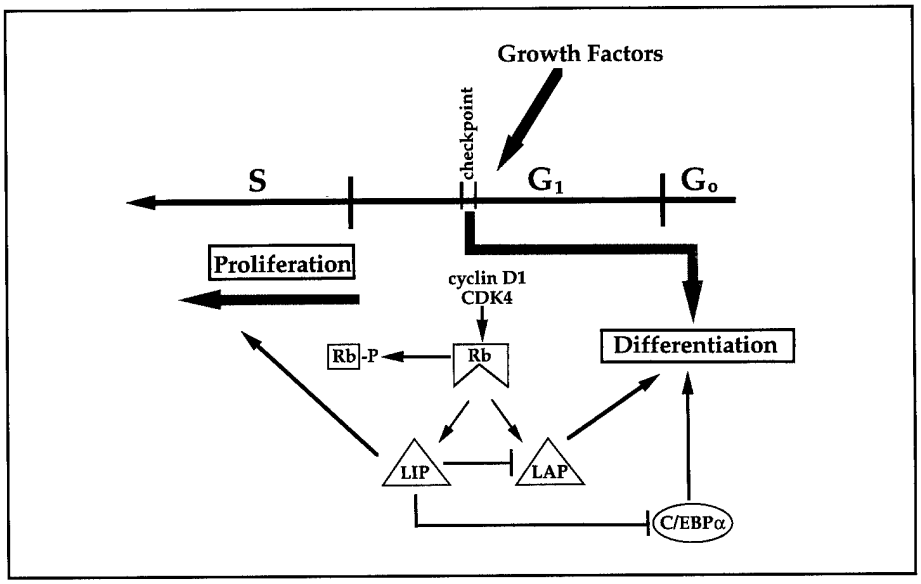

Fig. 3. Hypothetical model for the role of CCAAT/enhancer-binding proteins (C/EBPs) in cell cycle progression. The interaction of retinoblastoma protein (Rb) with the C/EBPβ–liver-enriched activating protein (LAP—35 kd) and liver-enriched inhibitory protein (LIP—20 kd) isoforms may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland (*see* text for details). Studies in adipocytes have demonstrated that C/EBPβ and C/EBPδ are involved in early proliferative and differentiative processes, leading to the activation of C/EBPα, which then contributes to terminal differentiation by arresting adipocyte proliferation *(16,17).* In HepG2 hepatoma cells, C/EBPβ-LAP has been reported to inhibit cell cycle progression before the G_1/S boundary, and this effect can be antagonized by expression of the dominant-negative LIP isoform, thereby promoting cell proliferation *(11).* In the rat mammary gland, C/EBPα expression is highest at lactation, when mammary epithelial cells undergo terminal differentiation, and C/EBPβ expression with an elevated ratio of LIP to LAP is highest during pregnancy, a period of lobuloalveolar proliferation *(12).* The ratio of LIP to LAP decreases almost 100-fold at the onset of lactation.

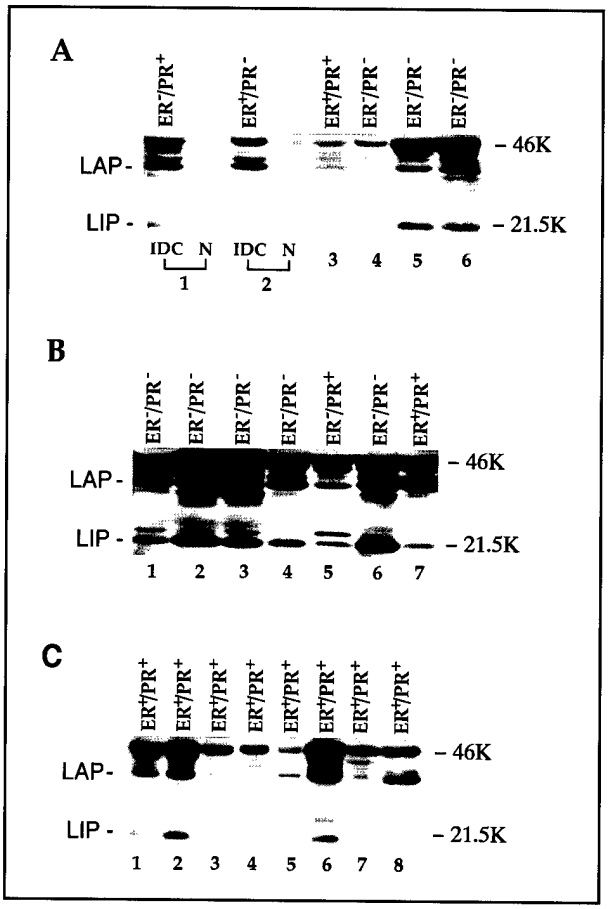

Fig. 1. Western blot analysis and enhanced-chemiluminescence (ECL) detection of CCAAT/enhancer-binding protein β (C/EBPβ) isoforms (liver-enriched activating protein [LAP—35 kd] and liver-enriched inhibitory protein [LIP—20 kd]) in human infiltrating ductal carcinomas. With the exception of lanes 1 and 2 in **panel A,** each lane represents the infiltrating ductal carcinoma from a different patient. Lanes 1 and 2 in **panel A** consist of an infiltrating ductal carcinoma (IDC) and its paired normal (N) tissue. One hundred micrograms of protein was analyzed per lane. The estrogen receptor (ER) and progesterone receptor (PR) status from each infiltrating ductal carcinoma is listed above the corresponding lane. The exposure time for **panel B** is not the same as that for **panel A** and **panel C,** and these western blot analyses were not all performed simultaneously. The variations in LIP levels on these ECL blots have been confirmed by use of the quantitative enhanced-chemifluorescence detection methodology described in the "Materials and Methods" section.

Table 1. Association of liver-enriched inhibitory protein (LIP) expression levels with steroid receptor status*

Receptor status	LIP expression level	
	High	Low or nondetectable
ER^-/PR^-	89% (8/9)	20% (6/30)
ER^+/PR^+	11% (1/9)	60% (18/30)
ER^-/PR^+	None	7% (2/30)
ER^+/PR^-	None	13% (4/30)

*Association of steroid receptor status with LIP overexpression in infiltrating ductal carcinomas. Of the 39 infiltrating ductal carcinomas examined, 14 (36%) were negative for estrogen and progesterone receptor (ER^-/PR^-), 19 (49%) were positive for estrogen and progesterone receptor (ER^+/PR^+), and six (15%) were either estrogen receptor negative and progesterone receptor positive (ER^-/PR^+) or estrogen receptor positive and progesterone receptor negative (ER^+/PR^-). High LIP values ranged from 5.2-fold above the standard to 0.5-fold below the standard. Likewise, low and nondetectable LIP values ranged from 0.3-fold below standard values to 0. Frequency analysis was performed by use of two-sided Fisher's exact test ($P = .0015$).

to be 1:2.4 (42.3% ± 6% [mean ± standard error]).

Two of the most frequently used prognostic indicators in breast cancer are tumor size and lymph node status. No association was observed between these two indicators and C/EBPβ-LIP levels. Unfortunately, the tissues analyzed from the Tissue Bank of The Methodist Hospital were biased toward tumors larger than 1 cm because of the inherent difficulties in banking tissue from smaller tumors. Consequently, tissue was analyzed from tumors ranging in size from 1.2 cm to 20 cm at the largest dimension. In addition, 22 patients had metastases to the lymph node, and the lymph node status for 11 patients was not determined. The sample size for the six lymph node-negative patients and the tumors smaller than 1 cm was, therefore, not large enough to draw any statistically significant conclusions.

To determine whether an association existed between genetic instability and LIP expression in the infiltrating ductal carcinomas, we compared levels of LIP expression in 38 of 39 tumor specimens with DNA ploidy. Nine (100%) of the nine tumors that expressed LIP at high levels were aneuploid. The tumors that expressed LIP at either low or nondetectable levels were more evenly distributed: 17 (59%) of 29 were aneuploid, whereas 12 (41%) of 29 were diploid ($P = .0356$, two-sided Fisher's exact test).

Because loss of ER expression is often associated with a poor clinical outcome *(9),* we next determined whether steroid receptor status in these tumors was associated with LIP expression. Of the 39 infiltrating ductal carcinomas examined, 14 (36%) of 39 specimens were negative for ER and PR (ER^-/PR^-), and 19 (49%) of 39 specimens were positive for ER and PR (ER^+/PR^+). Eight (89%) of nine ER^-/PR^- tumors expressed high levels of LIP, whereas only one (11%) of nine ER^+/PR^+ tumors exhibited high levels of LIP expression ($P = .0015$, two-sided Fisher's exact test) (Table 1).

Each tumor was graded blindly by use of the system of Elston and Ellis *(7),* which examines and scores the degree of tubule formation, the degree of nuclear pleomorphism, and the number of mitotic counts. All of the high LIP-expressing tumors (nine of nine) were classified as poorly differentiated (i.e., Elston–Ellis grade III) and highly proliferative tumors. In contrast, all three histologic grades (grade III—36.7% [11 of 30]; grade II—30% [nine of 30]; and grade I—33.3% [10 of 30]) were evenly distributed between the tumors expressing LIP at low or nondetectable levels ($P = .013$, two-sided Fisher's exact test). The proliferative fraction (determined by either MIB-1 immunohistochemistry and/or by DNA flow cytometry) also displayed an association with LIP expression. In the infiltrating ductal carcinomas that contained high LIP levels, eight (89%) of nine specimens contained a high fraction of proliferative cells, but only eight (30%) of 27 of the low or nondetectable LIP-expressing tumors contained a high fraction of proliferative cells ($P = .0046$, two-sided Fisher's exact test).

Although it was not possible to localize LIP expression to specific cell types, immunocytochemical staining was performed on a limited number of the infiltrating ductal carcinomas and their surrounding tissue to determine which cells expressed the C/EBPβ isoforms. These results were consistent with those obtained from the previous western blot analyses and revealed strong C/EBPβ expression in pleomorphic nuclei of grade III, ER^-/PR^- tumors (Fig. 2, A) and weaker C/EBPβ expression in the nuclei of grade I, ER^+/PR^+ tumors (Fig. 2,

affects Rb activity is not known. This interaction may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland and supports the hypothesis that the ratio of C/EBPβ isoforms may play a role in the control of cell cycle progression. We, therefore, propose that increased LIP expression may inhibit terminal differentiation and provide a selective growth advantage facilitating tumor progression (Fig. 3). Support for this hypothesis comes from the observations that the ratio of LIP to LAP is regulated during proliferative phases of both liver and mammary gland development *(5,11,12)*.

One caveat that must be considered in the interpretation of the foregoing results is the cellular heterogeneity that exists between breast tumors of the same type. For example, when infiltrating ductal carcinomas are compared, ER and PR levels may vary considerably in cells within a given tumor *(13,14)*. The degree of desmoplasia, lymphovascular invasion, and necrosis can also vary from patient to patient. Homogenization of tumor specimens for western blot or steroid receptor analyses can result in a misleading "dilutional" effect and does not permit direct cellular comparisons. This heterogeneity may be partly responsible for the variations observed in the LAP and LIP levels between infiltrating ductal carcinomas and tempers the associations of LIP expression levels with steroid receptor status and DNA ploidy. Furthermore, these studies do not permit an evaluation of ratios of LIP to LAP within individual cells of differing proliferative potentials within these tumors.

Mammary tumorigenesis is thought to result from multiple molecular changes, such as the activation of oncogenes by mutation, the overexpression of growth factors and their receptors, and/or the inactivation of tumor suppressor genes. It has been proposed that multiple molecular changes lead to a malignant phenotype, whereas, fewer "hits" are necessary for a benign tumor *(15)*. Our study suggests that overexpression of the naturally occurring, dominant-negative transcription factor C/EBPβ-LIP may be one of the molecular events that predisposes breast cells to a selective growth advantage, resulting in progression of infiltrating ductal carcinomas; however, additional tumors need to be examined to confirm our hypothesis.

These studies do not answer the question of whether overexpression of LIP, in the most advanced, aggressive tumors, facilitates tumor progression or if overexpression is simply a result of increased proliferation. These type of functional studies cannot be performed with patient biopsy specimens but require the use of animal or cell culture models. In this regard, we have recently generated transgenic mice that selectively express high levels of LIP in cells of the mammary gland (Zahnow CA, Rosen JM: unpublished observations). Studies are also in progress to determine the effects of regulatable LIP expression on cell cycle progression in mammary epithelial cells. These types of experiments will be required to determine if LIP expression is a cause or an effect of mammary tumorigenesis. Notwithstanding this determination, LIP expression may provide a useful additional marker for the identification of breast tumors in patients with a poor clinical outcome.

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Notes

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C/EBPβ-LIP Plays a Significant Role in Mammary Gland Proliferation

Cynthia A. Zahnow and Jeffrey M. Rosen

The CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors which regulate the expression of genes involved in proliferation and terminal differentiation. The gene encoding C/EBPβ is transcribed into a single mRNA which is translated via a leaky ribosome scanning mechanism, resulting in the synthesis of 2 principal isoforms in the mammary gland designated liver-enriched activating protein (LAP2-39kD) and the liver-enriched inhibitory protein (LIP-20kD). LIP functions as a dominant negative, and when dimerized with other C/EBP family members, suppresses the transcriptional activation of genes involved in differentiation. Because of an increased DNA affinity of the LIP isoform, this inhibition can occur even at sub-stoichiometric ratios of LIP/C/EBP. Consequently, an increase in LIP levels may inhibit terminal differentiation and lead to proliferation. Consistent with this hypothesis, LIP expression is elevated in a series of mouse and rat mammary tumors, but not hyperplasias, suggesting a role for this transcription factor in rodent mammary neoplasia (Raught *et al.*, Cancer Res:56, 1996). These preliminary data, as well as the reported observation that C/EBPβ can directly interact with the retinoblastoma protein (Rb), a regulator of cell cycle progression and a tumor suppressor gene product often mutated in breast cancer, has prompted us to investigate the role of C/EBPβ in normal mammary gland development and breast cancer.

We previously reported that LIP is overexpressed in human infiltrating ductal carcinomas and that this expression is highly associated with tumors which are aneuploid, estrogen and progesterone receptor negative, poorly differentiated and highly proliferative (Zahnow *et al.*, JNCI:89, 1997). These correlative studies do not directly address the role that LIP plays in tumorigenesis. Consequently, to assess the functional importance of LIP, we analyzed the effects of LIP overexpression in both an *in vitro* cell culture system and an *in vivo* transgenic mouse model. Expression of LIP in TM3 mammary epithelial cells resulted in a loss of contact inhibition, foci formation and reentry of the cells into the cell cycle. The proliferation rate during late confluence, as determined by percent of BrDU incorporation, was 11 times that of the non-LIP expressing clones.

The functional importance of LIP was also investigated in transgenic mice which overexpress LIP selectively in the mammary gland under the control of the whey-acidic protein (WAP) regulatory sequences. The WAP promoter preferentially targets high levels of transgene expression to mammary epithelial cells beginning at mid-pregnancy and extending to mid-lactation. Preliminary characterization of the phenotype associated with LIP overexpression during mid-lactation revealed an increased stromal compartment in many of the glands from three separate transgenic lines. Numerous lobules contain alveoli with collapsed or condensed lumens and irregularly shaped epithelial cells characterized by an altered nuclear to cytoplasmic ratio and abnormal secretions. Many ducts in the lactating gland are dysplastic as evidenced by the presence of numerous intra-ductal fingerlike projections and infoldings. Additionally, in the multiparous, involuted gland, there are multiple hyperplastic, epithelial cell pockets or clusters. Despite these abnormalities the mice appear to lactate normally and have not developed tumors to date. In summary, using a combinatorial approach of correlative clinical observations, and functionality studies using cell culture and transgenic animal models we have determined that C/EBPβ-LIP is an important regulator of proliferation in the mammary gland. Additional studies are underway to determine what role this proliferative response may play in tumor growth or progression .

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Keywords: C/EBPß isoforms, knockout mice, progesterone receptor

THE ROLE OF C/EBPs IN MAMMARY GLAND DEVELOPMENT Jeffrey M Rosen[1], Tiffany N Seagroves[1], Cynthia A Zahnow[1], and Shannon L Wyszomierski[1]. [1]Cell Biology, Baylor College of Medicine, Houston, TX.

The CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors containing highly conserved, basic leucine zipper motifs at their carboxy-termini, which mediate dimerization and DNA binding. Both activating(LAP, liver activating protein) and inhibitory(LIP, liver inhibitory protein) C/EBPß isoforms are differentially expressed throughout mammary gland development and in breast cancer, and interact with binding sites within the hormonally-regulated ß-casein promoter. These C/EBPß isoforms play important roles in both the repression of ß-casein gene expression and activation by lactogenic hormones. Transient transfection of the C/EBPß-LIP dominant negative isoform resulted in the repression of both the basal activity and inhibition of prolactin-induced ß-casein promoter activity. In contrast, transfection of the C/EBPß-LAP isoform in the presence of the glucocorticoid receptor and STAT 5 resulted in increased activation of the ß-casein promoter by lactogenic hormones. Altered expression of these C/EBPß isoforms also appears to influence hormonally-regulated mammary epithelial cell proliferation and terminal differentiation. C/EBPß LIP expression resulted in a loss of contact inhibition and reentry into the cell cycle in stably transfected TM3 mammary epithelial cells. C/EBPß LIP also is often overexpressed in a subset of human breast cancers that are estrogen(E) and progesterone(P) receptor negative, high S phase and aneuploid. Analysis of C/EBPß knockout(KO) mice has revealed its importance in ductal morphogenesis, lobuloalveolar development and functional differentiation in the mammary gland. These effects appear to be cell autonomous and were observed in KO mammary epithelium transplanted into the cleared mammary fat pad of wildtype mice. Both gene arrays and subtractive suppressive hybridization have been employed to identify downstream target genes for C/EBPß in the mammary gland. Surprisingly, the expression of many of these gene targets actually increased in E + P-treated KO mice suggesting that C/EBPß isoforms may play a repressive role in mammary gland development. One of the identified repressed genes was the progesterone receptor(PR). PR expression by both Northern blot and immunohistochemical analyses increased approximately 3-fold in E + P treated mice. This increase correlated with decreased proliferation and lobuloalveolar development observed in the E + P-treated C/EBPß KO mice. These studies support a new model of how P might influence cell proliferation during mammary gland development, and illustrate the importance of C/EBPß isoforms in normal mammary gland development and breast cancer. Supported by grants CA16303 from the National Institutes of Health and DAMD17-96-1-6086 from the U.S. Army.

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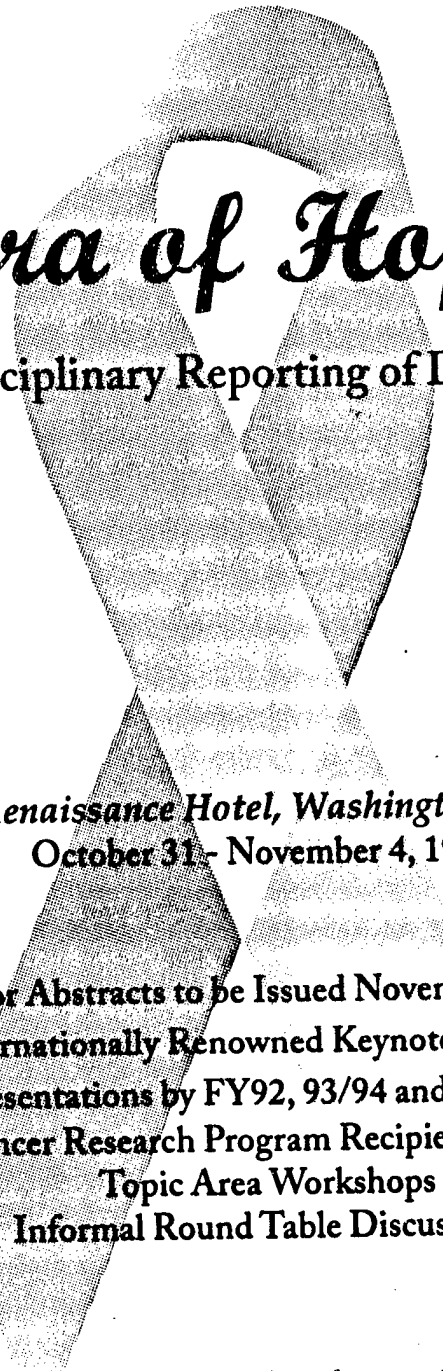
C/EBPβ-LIP: A Naturally-Occurring, Dominant-Negative Transcription Factor, and Its Role in Breast Cancer. Cynthia A. Zahnow[1], Pamela Younes[2], Rodolfo Laucirica[2], and Jeffrey M. Rosen[1]. [1]Department of Cell Biology, Baylor College of Medicine, and [2]Department of Pathology, The Methodist Hospital, Houston, Texas 77030.

Tumorigenesis often results from the failure of cells to maintain a balance between proliferation, terminal differentiation, and programmed cell death. The C/EBP family of DNA binding proteins may play a pivotal role in maintaining this balance by regulating the expression of genes involved in terminal differentiation. In general, C/EBPα is predominantly expressed at high levels in terminally differentiated, growth arrested cells and the C/EBPβ isoforms are expressed at high levels in actively proliferating cells. The gene encoding C/EBPβ is transcribed into a single mRNA. Translation of this mRNA occurs from 3 different in-frame AUGs via a leaky ribosome scanning mechanism, resulting in the synthesis of 3 proteins: liver-enriched activating proteins (LAP1-45kd, LAP2-39kd) and the liver-enriched inhibitory protein (LIP-20kd). LIP functions as a dominant negative, and when dimerized with other C/EBP family members, suppresses the transcriptional activation of genes involved in differentiation. Because of an increased DNA affinity of the LIP isoform, this inhibition can occur even at sub-stoichiometric ratios of LIP/C/EBP. Consequently, an increase in LIP levels may inhibit terminal differentiation and lead to uncontrolled proliferation and tumor growth. Consistent with this hypothesis, LIP expression is elevated in a series of mouse and rat mammary tumors, but not hyperplasias, suggesting a role for this transcription factor in mammary neoplasia (Raught *et al.*, Cancer Res.: 56, 1996). These preliminary data, as well as the reported observation that C/EBPβ can directly interact with the retinoblastoma protein (Rb), a regulator of cell cycle progression and a tumor suppressor gene product often mutated in breast cancer, has prompted us to investigate the role of C/EBP in human breast cancer. To test this hypothesis, we have examined LIP expression in the infiltrating ductal carcinomas (IDCs) from 39 women.

Frozen tissues were homogenized in RIPA and analyzed by Western blot analysis. LIP was detectable in 69% ($p<0.0008$) of the tumors. In contrast, LIP was not detected in "normal" breast tissue resected outside of the tumorigenic margins. Because the level of LIP expression differed amongst the various IDCs, we investigated whether a relationship existed between LIP expression and the degree of tumor differentiation or aggressiveness. Prognostic indicators such as, DNA ploidy, ER/PR status, and histological grade were thus correlated with LIP levels. Eighty one percent of the tumors expressing LIP were aneuploid, while the remaining 19% were diploid ($p<0.0004$). Of the 39 IDCs examined, 36% were ER-/PR-, and 49% were ER+/PR+. Ninety three percent of the ER-/PR- tumors expressed high levels of LIP, whereas, only 47% of the ER+/PR+ tumors exhibited LIP expression and this was at low to nondetectable levels ($p<0.006$). Each tumor was graded using the Elston/Ellis system which examines and scores the degree of tubule formation, the degree of nuclear pleomorphism and the number of mitotic counts. The poorest differentiated, most highly proliferative (grade III) Elston/Ellis tumors correlated with high levels of LIP expression. The better differentiated, less aggressive grade II and I tumors were associated with reduced LIP expression. These results were consistent with our immunocytochemical staining which revealed strong C/EBP expression in pleomorphic nuclei. Thus, LIP expression is highest in the tumors with the poorest prognosis. The naturally occurring, dominant negative, C/EBP-LIP may play an important role in regulating the switch between terminal differentiation, proliferation, and tumorigenesis in the mammary gland. The mechanisms responsible for the selective regulation of LIP expression and its functional consequences are currently under investigation. (Supported by DAMD 17-96-1-6086)

Department of Defense
Breast Cancer Research Program

Preliminary Announcement



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C/EBPβ-LIP: A NATURALLY-OCCURRING, DOMINANT-NEGATIVE TRANSCRIPTION FACTOR AND ITS PUTATIVE ROLE IN BREAST CANCER

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Tumorigenesis often results from the failure of cells to maintain a balance between proliferation, terminal differentiation, and programmed cell death. The C/EBP family of DNA binding proteins may play a pivotal role in maintaining this balance by regulating the expression of genes involved in terminal differentiation. In the mammary gland, C/EBPβ isoforms are expressed at high levels during pregnancy, but C/EBPα is predominantly expressed in the terminally differentiated, growth arrested cells of lactation. The gene encoding C/EBPβ is transcribed into a single mRNA. Translation of this mRNA occurs from 3 different in-frame AUGs via a leaky ribosome scanning mechanism, resulting in the synthesis of 3 proteins: liver-enriched activating proteins (LAP1-45kD, LAP2-39kD) and the liver-enriched inhibitory protein (LIP-20kD). LIP functions as a dominant negative, and when dimerized with other C/EBP family members, suppresses the transcriptional activation of genes involved in differentiation. Because of an increased DNA affinity of the LIP isoform, this inhibition can occur even at sub-stoichiometric ratios of LIP/C/EBP. Consequently, an increase in LIP levels may inhibit terminal differentiation and lead to uncontrolled proliferation and tumor growth. Consistent with this hypothesis, LIP expression is elevated in a series of mouse and rat mammary tumors, but not hyperplasias, suggesting a role for this transcription factor in rodent mammary neoplasia (Raught *et al.*, Cancer Res:56, 1996). These preliminary data, as well as the reported observation that C/EBPβ can directly interact with the retinoblastoma protein (Rb), a regulator of cell cycle progression and a tumor suppressor gene product often mutated in breast cancer, has prompted us to investigate the role of C/EBPβ in normal breast development and breast cancer. We have addressed this question through analysis of C/EBPβ expression in human breast cancer (infiltrating ductal carcinomas, IDCs) and correlation of LIP expression with various prognostic indicators. Additionally, the mechanisms responsible for the selective

Keywords: C/EBPβ, Breast Cancer, Cell Cycle, Transgenic Mice, Transcription Factor

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regulation of LIP expression and its functional consequences are currently being investigated in transgenic mice which overexpress C/EBPβ-LIP selectively in their mammary glands.

Frozen breast cancer tissues were homogenized in RIPA buffer and analyzed by both ECL and ECF Western blot analyses. LIP was expressed at high to moderate levels in 51% of the tumors. Because the level of LIP expression differed amongst the various IDCs, we investigated whether a relationship existed between LIP expression and the degree of tumor differentiation or aggressiveness. Prognostic indicators such as DNA ploidy, ER/PR status, and histological grade were thus correlated with LIP levels. Ninety percent (18/20) of the tumors which expressed LIP at high to moderate levels were aneuploid, while the remaining 10% (2/20) were diploid. Whereas, the tumors which expressed LIP at lower levels were more evenly distributed between aneuploid 47% (8/17) and diploid 53% (9/17). Of the 39 IDCs examined, 36% were ER^-/PR^-, and 49% were ER^+/PR^+. Ninety three percent (13/14) of the ER^-/PR^- tumors expressed high to moderate levels of LIP, whereas, only 26% (5/19) of the ER^+/PR^+ tumors exhibited high to moderate levels of LIP expression. Each tumor was graded using the Elston/Ellis system which examines and scores the degree of tubule formation, the degree of nuclear pleomorphism and the number of mitotic counts. The poorest differentiated, most highly proliferative (grade III) Elston/Ellis tumors correlated with high levels of LIP expression. The better differentiated, less aggressive grade II and I tumors were associated with reduced LIP expression. These results were consistent with our immunocytochemical staining which revealed strong C/EBPβ expression in pleomorphic nuclei. Thus, LIP expression is highest in the tumors of the patient's with the poorest prognosis. The naturally occurring, dominant negative, C/EBPβ-LIP may play an important role in regulating the switch between terminal differentiation, proliferation, and tumorigenesis in the mammary gland.

These studies do not answer the question of whether overexpression of LIP in aggressive breast tumors facilitates tumor progression or is simply a result of the increased proliferation. To assess the functional importance of LIP expression, we have recently generated transgenic mice which overexpress LIP under the control of the whey-acidic protein (WAP) regulatory sequences. The WAP promoter preferentially targets high levels of transgene expression to mammary epithelial cells beginning at mid-pregnancy and extending to mid-lactation. Preliminary characterization of the phenotype associated with LIP overexpression during lactation revealed increased stromal tissue, which may be a result of the failure of the epithelial cells to completely grow out into the fat pad. Additionally, many lactating lobules contain alveoli with uncharacteristically small lumens and irregularly shaped epithelial cells. Further characterization will include an analysis of markers of cellular differentiation (β-casein, C/EBPα and p21) and cellular proliferation as well as an estimation of changes in apoptosis levels. Because tumorigenesis is the result of multiple genetic disruptions and is not usually due to a change in a single molecular event, these transgenic mice will be crossed with other transgenic mouse models including C/EBPβ–knockout mice and p53 Arg-His mutants. The p53 mutant does not alter mammary gland development and tumor formation seldom occurs, however; when exposed to a carcinogen or an altered growth factor signaling pathway, these mice develop genetic instability and accelerated mammary tumor growth. A cross with either of these models may help to determine whether C/EBPβ–LIP contributes to tumor progression and what signaling pathways are involved. It is hoped that our combinatorial approach of correlative and functional studies will discern the role of C/EBPβ–LIP in breast cancer.

A PUTATIVE ROLE FOR C/EBPβ-LIP IN BREAST CANCER

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During breast development, hormones promote changes in growth that occur during puberty, the menstrual cycle, pregnancy and lactation. Cell death must also occur in order for the breast to return to its original state following pregnancy or nursing. Tumor formation often results when breast cells cannot maintain a balance between growth and programmed cell death. The C/EBP family of proteins is regulated by hormones and plays a role in maintaining this balance. One of the C/EBP family members, C/EBPβ-LIP, promotes cellular growth and we have evidence that C/EBPβ-LIP is overproduced in rodent mammary gland tumors. This has led us to examine whether C/EBPβ-LIP plays a role in human breast cancer. We analyzed breast cancer tissue from 39 women and found C/EBPβ-LIP levels to be high in at least half of the tumors. Interestingly, the patients with the poorest prognosis also contained the highest levels of C/EBPβ-LIP in their tumors. This correlation does not directly address the question of C/EBPβ-LIP's role in breast cancer. To determine the function of C/EBPβ-LIP in normal and abnormal breast development we have engineered a mouse that overproduces C/EBPβ-LIP exclusively in its mammary gland. Preliminary characterization has revealed that the gland has not developed normally. Because breast cancer is the net result of multiple changes and is not normally due to a single change, we plan to breed these mice with others that have been genetically altered and have other changes associated with abnormal breast development. The mammary glands from these offspring will then be studied. Little is known about the role C/EBPβ-LIP may play in breast cancer and our research may thus provide insight into a new pathway that may be altered in breast cancer and thereby provide new strategies for treatment. C/EBPβ-LIP expression may prove to be a useful marker for the identification of aggressive breast tumors in patients with a poor clinical outcome.

Cynthia C. Zahnow

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A NOVEL ROLE FOR C/EBP IN BREAST CANCER. C. Zahnow, B. Raught, A. James, and J.M. Rosen. Baylor College of Medicine, Houston Texas, 77030.

Tumorigenesis often results from the failure of cells to maintain a balance between proliferation, terminal differentiation and programmed cell death. The hormonally regulated C/EBP family of DNA binding proteins may play a pivotal role in maintaining this balance by regulating the expression of genes involved in terminal differentiation. The C/EBPβ dominant negative isoform, LIP, dimerizes with other C/EBP proteins and inhibits their ability to transactivate genes involved in differentiation. We are investigating whether an increase in LIP levels or activity may inhibit terminal differentiation and lead to uncontrolled proliferation and tumorigenesis in the mammary gland.

Our preliminary western blot analyses have demonstrated that LIP levels are elevated in rodent and human mammary carcinomas. LIP expression was observed in mammary tumors of different etiologies, including primary tumors induced in transgenic mice by the overexpression of TGFα and Wnt-1 in both $p53^{+/+}$ and $p53^{-/-}$ backgrounds, and in carcinogen-induced rat mammary carcinomas, but was not observed in a number of different primary tumors in transgenic and knockout mouse models, including lens, ovary, lung and prostate. Additionally, we have examined LIP expression in six human mammary carcinomas and have observed high LIP levels in three of the tumors. These three infiltrating ductal carcinomas were aneuploid, of high nuclear grade, estrogen- and progesterone-receptor negative and were highly proliferative. LIP expression was not detectable in the control, non-tumorigenic, mammary tissue from these patients. These data suggest that elevations in LIP expression in breast cancer patients may be associated with a poor prognosis (Supported by NIH CA16303).

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